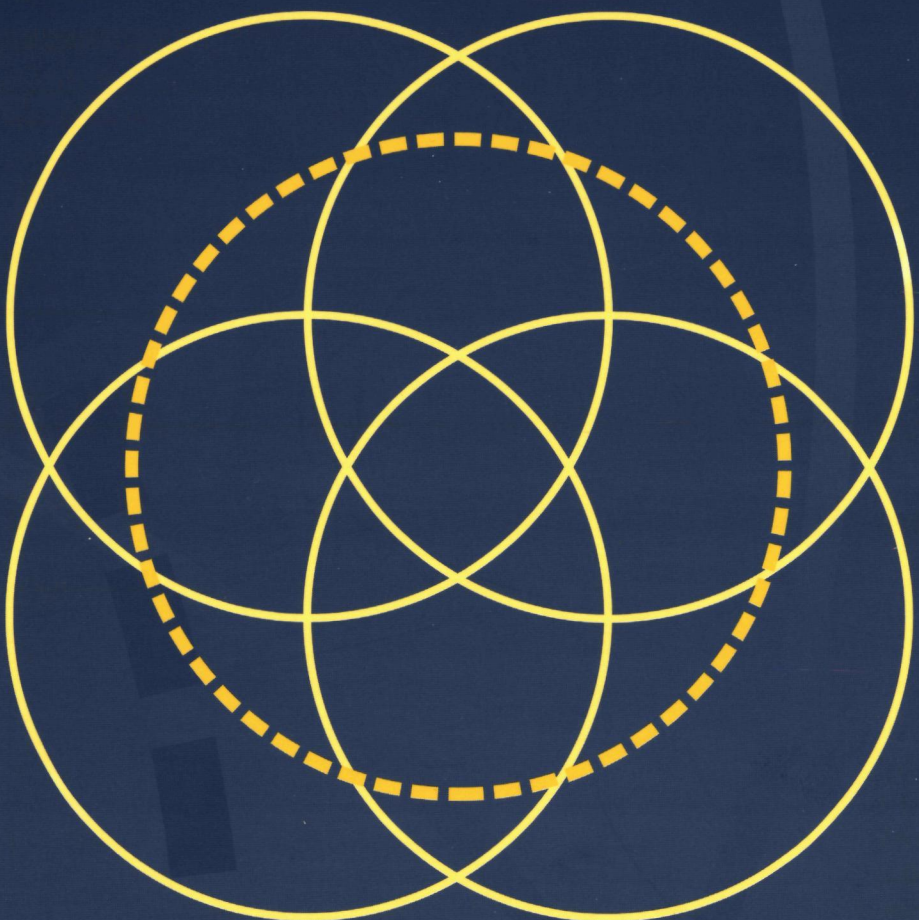
Familial combined hyperlipidemia

Metabolic and genetic aspects



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Voor Karin en Marieke

CONTENTS

ABBREVIATIONS

Apo	apolipoprotein
BMI	body mass index
CETP	cholesteryl ester transfer protein
FCH	familial combined hyperlipidemia
FFA	free fatty acids
HDL	high density lipoprotein
HtgL	hepatic triglyceride lipase
IDL	intermediate density lipoprotein
LDL	low density lipoprotein
Lp(a)	lipoprotein(a)
LpL	lipoprotein lipase
NEFA	non-esterified fatty acids
TG	triglycerides
VLDL	very low density lipoprotein

Chapter 1

General introduction and outline of the thesis

INTRODUCTION

Although our knowledge about etiology, pathophysiology and treatment of cardiovascular disease has increased enormously, it still remains the most frequent cause of death in western countries In 1992, 40% of all deaths (about 52 000 deaths) in the Netherlands was due to a form of cardiovascular disease The last 15 years numerous retrospective studies have provided accumulating evidence for the causal role of elevated plasma cholesterol in the origin of cardiovascular disease Only recently, also large scale prospective primary and secondary prevention trials indisputably showed a decrease in cardiovascular mortality and regression of angiographically defined atherosclerotic lesions by a reduction of plasma cholesterol levels (1-5) Furthermore, mounting evidence indicates that next to elevated plasma cholesterol, elevated concentrations of triglyceride-rich lipoproteins determine the cardiovascular risk as well (6-9)

Among the different lipid disorders, the most frequently occurring one, known as familial combined hyperlipidemia (FCH), has still kept lots of its secrets about etiology, pathophysiology and mechanism of associated premature atherosclerosis As a consequence, a specific marker, based on supposed specific metabolic abnormalities in FCH, and necessary for individual diagnosis, is lacking (10) Patients suffering from FCH frequently show only marginal elevated lipid levels Therefore, the observed disproportional increase in cardiovascular risk of these subjects must come from certain abnormalities in their lipid profiles, which is determined by overproduction of very low density lipoproteins (VLDL) (11,12) Besides VLDL overproduction, also an impaired physiological clearance of triglyceride-rich particles, both of exogenous and of endogenous origin (13), contributes to an increased pool of triglyceride-rich lipoproteins underlying the multiple lipid phenotype expression of FCH patients (11,14-16) Such a lipid profile is frequently associated with an unfavorable decrease in high density lipoprotein (HDL) concentration and a preponderance of atherogenic small dense low density lipoprotein (LDL) subfractions (17-21)

To have a chance to obtain a specific marker for the disorder FCH, both the etiology of the increased pool of apolipoprotein B100 containing triglyceride-rich lipoproteins as well as that of the preponderance of small dense LDL particles in FCH patients have to be elucidated Consequently, factors involved in VLDL-apoB100 overproduction and clearance of these particles, as well as metabolic influences of elevated plasma triglyceride levels on LDL subfraction distribution and possible genetic control on LDL density in FCH are of great interest

METABOLISM OF APOB100 CONTAINING LIPOPROTEINS

Once produced, VLDL is metabolically converted into LDL with persevation of apoB100. The increase in lipoprotein density during this conversion depends on constant triglyceride hydrolysis by lipoprotein lipase (LpL) and hepatic triglyceride lipase (22). Abnormal LpL activity is reported to be a major cause of impaired triglyceride-rich lipoproteins clearance in FCH (23-27). The metabolic role of HtgL is only partly understood. As has been reported, HtgL may determine LDL density (28-30).

Due to lack of apoE-mediated receptor uptake of triglyceride-rich apoB100 particles, patients suffering from familial dysbetalipoproteinemia exhibit an increased plasma cholesterol and triglyceride level. This is caused by a defective B/E receptor binding in case of homozygous apoE2/2. However, for complete phenotypic expression of this form of hyperlipidemia additional factors are needed. In general, apoE polymorphism determines a detectable fraction of the hypertriglyceridemia, in the presence of an apoE2 allele, and of the hypercholesterolemia in the presence of an apoE4 allele, in some forms of hyperlipidemia (31-36). Consequently, apoE polymorphism may play a role in lipid phenotypic expression of FCH as well.

The increased secretion rate of VLDL-apoB100 in FCH depends at least in part on free fatty acids (FFA) availability to hepatic cells, which are either released from adipocytes, intrahepatically stores, or are de novo synthesized from dietary carbohydrates (22,37). Insulin resistance is frequently associated with elevated plasma FFA concentrations due to an impaired postprandial FFA uptake by adipocytes which stimulates VLDL synthesis (38). Furthermore, insulin resistance is associated with an impaired ability of insulin to stimulate LpL (39,40). By measuring plasma FFA and insulin concentrations, it was suggested that insulin resistance may underly FCH (41-43).

FCH AND LDL HETEROGENEITY

Since the first reports by Hammond and Fisher, it has been recognized that LDL particles are markedly heterogeneous in physical and chemical properties, which includes particle size, buoyant density, chemical composition, surface electrical charge and hemodynamic behaviour (44-50). Recently it was demonstrated that small dense LDL subspecies exhibit an enhanced susceptibility to oxidative modification compared with buoyant subfractions, and may therefore be more atherogenic than buoyant subspecies (51-53). The hypothesis of oxidative modification preceeding atherogenesis is supported by the demonstration of oxidized LDL in vivo and autoantibodies against oxidized LDL particles (54-56). Differences in oxidative susceptibility are explained by variability in

fatty acid and antioxidant contents (53), whereas specific different physiochemical and structural properties of the LDL subfractions in FCH may contribute (46).

The origin of LDL heterogeneity and the cause of LDL subfraction profiles predominated by small dense LDL particles is only partly understood. Most likely, LDL heterogeneity results from a large variety of metabolic, genetic as well as environmental factors. Intravascular formation of LDL subfractions involves the conversion of VLDL precursors. Exchange of LDL cholesteryl ester for VLDL triglycerides, mediated by the cholesteryl ester transfer protein (CETP), would result in an enrichment of the LDL with triglycerides (57,58). The subsequent triglyceride hydrolysis by mainly hepatic triglyceride lipase would result in a decreased particle size (29,30,59,60). Although this theory explains the preponderance of small dense LDL particles in hypertriglyceridemia, it can not account for less frequently observed buoyant LDL particles in this condition, or the predominance of small dense LDL in the absence of hypertriglyceridemia. Therefore, also direct hepatic secretion of dense IDL or LDL, and small dense LDL selectively originate from certain VLDL precursors are hypothesized (61-63).

Recently, genetic influence on individual LDL subfraction distribution investigated by segregation analysis has been reported (64-66). These studies suggested the presence of susceptibility genes determining the LDL subfraction profile, which may have connections to metabolic pathways involved in hyperlipidemia. Therefore, assessing genetic influence on the LDL subfraction profile of FCH patients may reveal metabolic defects underlying FCH.

OUTLINE OF THE INVESTIGATIONS

Since 1973, FCH has been recognized as autosomal inheritable primary lipid disorder. The recognition of individual patients with familial combined hyperlipidemia who are prone to develop premature cardiovascular disease is, however, complicated by the absence of one or more specific markers for the disorder. Therefore, putting a proper diagnosis still necessitates laborious and time consuming family investigation. Furthermore, the mechanism by which FCH patients are more prone to premature cardiovascular disease is only partly understood and may be related to the increased pool of triglyceride-rich lipoproteins and the predominance of small dense LDL particles characteristic for the disorder. The objective of the studies presented in this thesis is to establish in FCH patients the role of several metabolic factors involved in the production and clearance of apoB100 containing lipoproteins in order to gain more insight into the etiology of FCH. Especially, the origin of LDL heterogeneity in FCH was investigated.

By performing family studies the impact of metabolic and genetic factors determining small dense LDL could be elucidated. Furthermore, a possible mechanism underlying the augmented prevalence of atherogenesis in FCH due to predominance of small dense LDL was studied.

In detail, blood samples and required data of 687 family members from 40 well-defined FCH families were collected. To investigate features of metabolic coherence in FCH, the effects of two different hypolipidemic drugs on apoB100 containing lipoproteins were evaluated in 81 FCH patients. This allowed us to establish in a subgroup of this study the metabolic relations between different lipids or lipoprotein fractions and LDL heterogeneity. In addition, the susceptibility of LDL to in vitro oxidative modification, both before and after treatment was determined to evaluate possible consequences for the atherogenic potential of this lipid fraction in FCH.

As outlined above LpL and ApoE are determinants of the catabolism of triglyceride-rich particles. Hydrolysis of lipoproteins by LpL results in remnant particles and apoE faciliates a receptor-mediated uptake of lipoproteins. The role of apoE polymorphism on the lipid phenotypic expression and the distribution of LDL subfractions was investigated in all FCH families. Furthermore, the consequences of impaired LpL activity due to mutations in the LpL gene were investigated. In 3 large families of probands exhibiting the recently discovered LpL Asn291→Ser mutation, the relationship between genotype and phenotype could be established.

Insulin resistance is reported to be associated with typical features of FCH, like VLDL overproduction, an impaired postprandial clearance of triglyceride-rich lipoproteins, and a predominance of small dense LDL. Furthermore, hyperinsulinemia per se is also directly associated with cardiovascular disease. To investigate whether insulin resistance plays a role in the etiology of FCH, the insulin sensitivity of 11 non-obese FCH patients and 11 first-degree relatives was compared directly by the euglycemic hyperinsulinemic clamp test. In this study, differences in insulin induced vasodilation which may in part be responsible for diminished glucose uptake were measured as well.

As recently postulated, enhanced susceptibility of small dense LDL particles to oxidative modification would explain the atherogenic potential of these particles. Resistance against in vitro oxidation may be related with the presence of lipophilic antioxidants. In the FCH families, possible disorder-related differences in susceptibility towards oxidative modification, expected by differences in the presenting LDL subfraction profile, were investigated. In 35 FCH family members with either a predominance of dense or a predominance of buoyant LDL particles, the oxidizability of total LDL was

determined and a relation to LDL density and the redox status of the antioxidant ubiquinol-10, reflecting the actual in vivo LDL oxidation status, was observed.

To investigate whether genetic factors influence the LDL subfraction profile in FCH, a segregation analysis using a continuous variable K to describe individual LDL subfraction profiles was performed in the FCH families. The presence of small dense LDL shows strong correlation with plasma triglycerides and plasma apoB. Consequently, evidence for a major locus controlling the observed LDL heterogeneity in these families may be due to genetic factors directly responsible for hypertriglyceridemia or hyperapobetalipoproteinemia. Cholesterol concentrations in ApoB100 containing lipoproteins were found to correlate well with plasma apoB100 levels both in normolipidemic and hyperlipidemic relatives. Therefore, also a segregation analysis on plasma apoB100 was performed to study inheritance of this lipid parameter in FCH families. In order to investigate whether observed genetic models for inheritance of LDL subfraction profiles and plasma apoB100 represent different genetic mechanisms, a final analysis was performed. In this segregation analysis the LDL subfraction profiles were corrected for the predicted apoB100 level genotype influence.

All these studies revealed arguments for considering the presence of small dense LDL as a biochemical marker for the lipid disorder FCH.

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Chapter 2

Metabolic and genetic aspects of Familial Combined Hyperlipidemia with emphasis on LDL heterogeneity

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INTRODUCTION

The relationship between elevated plasma cholesterol and the risk of coronary artery disease is definitively established now (1-5). Accumulating evidence indicate that the total amount of triglyceride-rich lipoprotein particles, i.e. chylomicron remnants, very low density lipoproteins (VLDL) and intermediate density lipoproteins (IDL) determine the risk to develop cardiovascular disease as well (6-8). This would explain the extending benifit of cholesterol-lowering therapy observed in the majority of patients with coronary disease who have only marginally elevated plasma cholesterol levels, but may exhibit other lipid abnormalities (4,9).

In patients suffering from familial combined hyperlipidemia (FCH) elevated levels of triglyceride-rich lipoproteins mainly determine the presenting lipid phenotype. Because FCH appeared to be the most common form of hyperlipidemia in young survivors of myocardial infarction (10,11), causing an estimated 10% of all premature coronary heart disease (12,13), recent research has been focussed on the pathophysiological mechanism underlying premature atherogenesis in FCH. In this review, hypotheses about the metabolic and genetic basis of FCH and its related entities, as well as the origin of LDL heterogeneity associated with these lipid disorders will be discussed.

PHENOTYPIC DIAGNOSIS OF FCH

In 1973, FCH was first described by Goldstein et al. (10) and Rose et al. (12), shortly followed by others (11,14,15), as a new autosomal dominant inherited lipid disorder, characterized by elevated plasma cholesterol and triglyceride levels in first-degree relatives, and strongly associated with premature cardiovascular disease. At that time, the recognition of FCH confounded the previously formulated Fredrickson classification of hyperlipoproteinemias by the presence of first-degree relatives exhibiting different lipid phenotypes within one single family.

Because there is no specific marker for the disorder while the lipid phenotypic expression among affected individuals may show some variation in time, the diagnosis is necessarily based on family investigation to demonstrate a so called 'mixed hyperlipidemia' with either hypercholesterolemia, hypertriglyceridemia, or combined hyperlipidemia in first-degree relatives. Criteria supporting the FCH diagnosis are presented in Table 1. Nowadays it is common sense that all main inclusion criteria, in the abcense of all exclusion criteria, should be met for a true diagnosis, whereas the diagnositic value of the mentioned additional inclusion criteria is still under debate.

Table 1. Inclusion and exclusion criteria supporting the diagnosis familial combined hyperlipidemia.

Main inclusion criteria:
- Presence of a multiple type hyperlipidemia in first-degree relatives of a single family comprising hypertriglyceridemia, hypercholesterolemia, and combined hyperlipidemia, as defined by fasting plasma cholesterol and/or plasma triglyceride concentrations above the 90th percentile for age and gender
- Autosomal dominant inheritance of the hyperlipidemia
- Presence of premature atherosclerosis (before age of 60 years) in first-degree relatives
Additional inclusion criteria:
- An elevated total plasma apolipoprotein-B concentration
- A LDL subfraction profile predominated by small dense LDL particles
- Manifestation of the hyperlipidemia in adolescence
Exclusion criteria:
- Presence of any form of xanthoma in first-degree relatives
- Presence of a secondary cause for the hyperlipidemia in affected relatives
- Presence of the Apo $\epsilon 2/\epsilon 2$ genotype in first-degree relatives

Although FCH patients frequently exhibit elevated plasma apo B concentrations (13,16) when compared with their normolipidemic relatives, the interpretation of total plasma apolipoprotein-B (apo B) levels as diagnostic criterium is still open for discussion. A plasma apo B100 level above 130 mg/dl, standardized upon the radioimmunoassay method of the International Union of Immunological Societies, may contribute to define FCH patients (17,18). Considering that the lipid to protein ratio of VLDL and LDL particles is relatively constant even in FCH patients (13,19,20), total plasma apo B could be derived from the strong correlation that appeared to exist between VLDL plus LDL cholesterol and plasma apo B (16). Therefore, plasma apo B strongly correlates with the presented FCH lipid phenotype based on elevated VLDL and/or LDL concentrations (16).

For unclear reasons, the manifestation of hyperlipidemia in childhood, as frequently observed in familial hypercholesterolemia, does rarely occur in FCH (21). However, hyperapobetalipoproteinemia, a feature associated with FCH, has been detected in children of parents with premature cardiovascular disease (22,23).

FCH AND ITS RELATED LIPID PHENOTYPES

Initially FCH was thought to be inherited as a single gene disorder with a major effect on triglyceride levels (10). Recently, again evidence for a major gene effect on triglyceride levels was provided by complex segregation analysis in British FCH families (24). Whereas this supposed gene mutation has still not been located, other studies indicated that a variety of metabolic and biochemical defects predispose for the FCH phenotype, suggesting that the genetic basis of this trait is heterogeneous and may even involve several defects in one family.

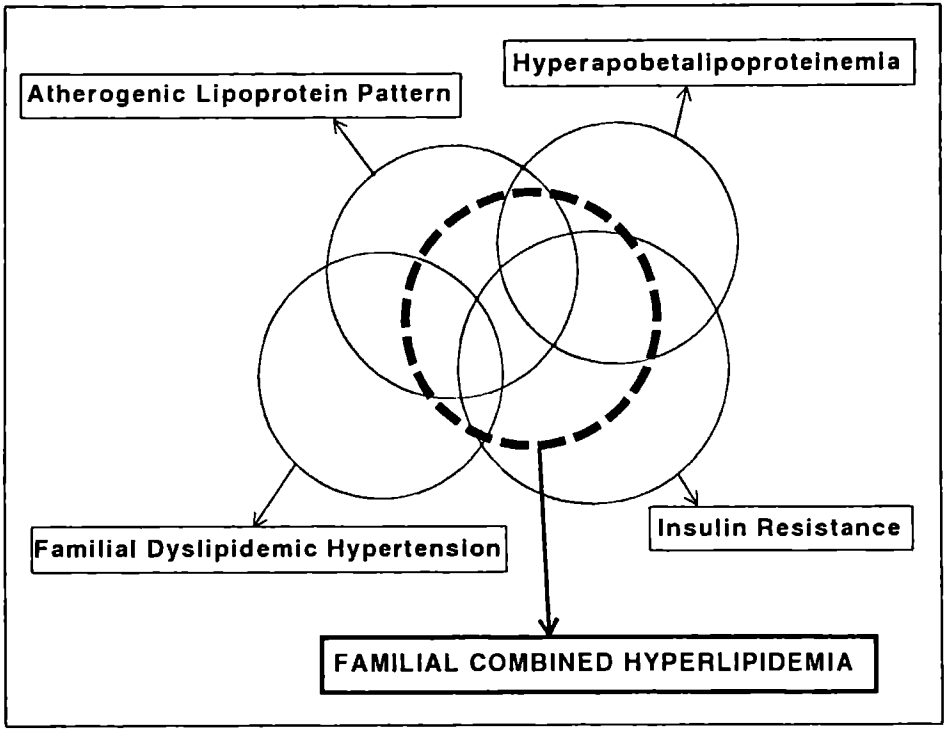

Figure 1: Overlapping characteristics of four entities with key features of the FCH phenotype, which may contribute to the complete picture of FCH. (freely adapted from Kwiterovich P.O.J , Curr. Opin Lipidol. (1993)4:133-143).

According to these reports FCH may be considered more like a 'syndrome', showing

overlapping characteristics with other entities such as (i) hyperapobetalipoproteinemia (hyperapoB) defined by a normal LDL cholesterol level with an increased LDL protein (apo B) content (25); (ii) the 'atherogenic lipoprotein phenotype' (ALP) characterized by increased triglyceride and apo B levels, decreased HDL levels and a predominance of small dense LDL (26); (iii) familial dyslipidemic hypertension (FDH) a syndrome of mixed lipid abnormalities resembling the FCH phenotype, associated with mild hypertension (27); and (iv) the insulin resistance syndrome associated with increased VLDL production and impaired clearance of triglyceride-rich particles, also key features of FCH (28,29) (Figure 1).

PATHOPHYSIOLOGY OF FCH

In general, FCH is thought to be caused by hepatic VLDL overproduction with or without impaired clearance of triglyceride-rich lipoproteins (30,31). Since no single metabolic defect detected sofar could fully account for the FCH phenotype, it was hypothesized that a number of defects are involved. Considering these defects, it still has to be settled whether they are causal for the disorder or are a regulatory consequence of an underlying metabolic defect. In general, circulating triglyceride-rich lipoproteins are of exogenous or endogenous origin. For better comprehension, these pathways are described more specific below.

The exogenous pathway

This pathway involves the transport of dietary lipids from the intestine to the liver by apo B48 containing chylomicrons. Due to the action of the enzyme lipoprotein lipase (LpL), activated by cofactor apo CII, fatty acids are liberated from chylomicrons and pass to the adipose tissue or skeletal muscle cells to be oxidized or stored. Reduced LpL activity due to LpL gene mutations has been reported repeatedly in subsets of FCH populations (32,33) and may result in impaired clearance of chylomicrons. For storage in adipocytes, free fatty acids (FFA) are intracellularly reesterified to triglycerides, a process that is mediated by the action of a basic protein called acylation stimulatory protein (ASP) (34). Due to impaired ASP activity, as reported in hyperapoB, a reduced rate of FFA uptake into adipocytes may result in an increased flux of FFA to the liver and consequently in an increased hepatic VLDL synthesis (35). After the release of FFA, the remainder chylomicron remnant particles are taken up by the liver via a specific remnant receptor that only recognizes apo E as ligand (36). A delayed clearance of atherogenic chylomicron remnants, possibly due to competition between chylomicrons and endogenous

VLDL for available LpL activity and competition for remnant receptor capacity, has been reported to exist in FCH patients (37). In the hepatocytes, all components of the remnants are hydrolyzed in the lysosomal compartment and a part of this material is re-used to form nascent VLDL particles entering the endogenous pathway. Intracellular increase of cholesterol in hepatocytes may increase plasma LDL cholesterol concentration because of hepatic LDL receptor down-regulation.

The endogenous pathway

This pathway involves the assembly of formed endogenous cholesterol and triglycerides in the core of VLDL followed by excretion into the circulation. In vitro studies show that in HepG2 cells intracellular triglyceride biosynthesis, but not the rate of cholesterol or cholesteryl esters determines the secretion rate of VLDL-apo B (38). The triglyceride biosynthesis itself depends on the availability of required FFA which are either released from adipocytes, intrahepatically stored, or converted from dietary carbohydrates (39). The release of required FFA from visceral adipocytes is mediated by the action of the enzyme hormone sensitive lipase (HSL) (40,41). Postprandial hyperinsulinemia plays a regulatory role because it inhibits the lipolytic effect of HSL to allow FFA uptake by adipocytes (42). FCH is associated with increased insulin resistance which would allow for increase of VLDL production by net increase of serum FFA (43,44). Without merging with lipids to allow the formation of a VLDL particle, nascent apoprotein B100 is degraded. This process is catalyzed by the action of microsomal triglyceride transfer protein (MTP), referring to its site of action in the hepatic endoplasmatic reticulum (45,46). Recently, abetalipoproteinemia, the metabolic 'opposite' of FCH, was found to be caused by MTP absence (47). Consequently, it has to be established whether MTP overexpression could also play a role in VLDL-apo B overproduction of FCH.

The continuous hydrolysis of core triglycerides in FFA by LpL converts VLDL into smaller apoprotein B100 containing VLDL remnants, IDL and LDL (39,48,49). Recent reports suggest that a heterozygous state for one of the mutations found in the LpL gene affecting its activity, may result in a lipoprotein pattern classified as FCH (32,33,50). However, it remains unclear whether this phenomenon is only more pronounced in hyperlipidemic subjects compared to normolipidemic individuals without an underlying metabolic defect. Accurate data about the prevalence of these mutations in different populations may help to interpret the observed influences. Increased apo CIII levels are associated with impaired clearance of triglyceride-rich lipoprotein due to direct inhibition

of LpL by apo CIII (51) Interestingly, linkage between the FCH phenotype and the AI/CIII/AIV gene cluster has been reported (52). However, this finding could not be confirmed by others, although several polymorphisms in the gene cluster were recently found to amplify the phenotypic expression in FCH (53).

A portion of small VLDL, i e , IDL, is catabolized after apoprotein E mediated binding to hepatic LDL or B/E receptors, which differ from the chylomicron remnant receptor The affinity for the B/E receptor depends on the apo E isoform (i.e., high for apo E3 and E4, but low for apo E2). A recent study on the effects of apo E polymorphism on presenting lipid phenotype in FCH suggested that differences in apo E isoform-related clearance may only contribute to the hyperlipidemia due to other defects (54) Further hydrolysis of triglycerides predominantly by hepatic triglyceride lipoprotein lipase (HtgL) processes remaining IDL into LDL particles which then mainly consist of cholesteryl esters and apoprotein B100 (55) Exchange of LDL cholesteryl esters with VLDL triglycerides mediated by cholesteryl ester transfer protein (CETP) activity determines in part the observed heterogeneity of LDL particles (56), as will be discussed later

FCH AND LDL HETEROGENEITY

Introduction

It has been recognized for a number of years that LDL particles are markedly heterogeneous in physical and chemical properties (57-61). In FCH, these properties of LDL are reported to be different from normal (19,62,63) The LDL subspecies in FCH are heterogeneous with a propensity toward small dense particles (13,19,64) The predominance of small dense LDL subfractions in FCH family members may not be fully explained by metabolic processing alone Direct still unclarified genetic influences on the distribution are proposed as well (16,65,66) It is only recently that also the relationship of qualitative features of LDL particles to cardiovascular disease has attracted considerable interest This interest was raised by reports that certain LDL subpopulations may be more atherogenic than others due to differences in susceptibility towards oxidative modification (58,67-70) However, major questions about origin, structural variation and biological function of LDL subspecies are still only partly understood

Identification and characterization of LDL subfractions

Since the first reports on measurements of LDL heterogeneity, two basically different techniques have been used to identify LDL subspecies (1) Nondenaturating gel

electrophoresis (GGE) of whole plasma or of isolated LDL, which separates several LDL subspecies based on differences in size (57); (ii) Density gradient ultracentrifugation (DCUG) of whole plasma, based on differences in density within the LDL subclass population (71,72). Nowadays, both techniques are widely used in large-scale studies to identify LDL heterogeneity.

Using GGE, most individuals are characterized by only two or three peaks or shoulders on the densitometric scan. Based on the location of these peaks on the gel, LDL subclass pattern can be classified dichotomously in a pattern A, predominantly characterized by large LDL particles, and a pattern B, predominated by small LDL particles (26,68). Recently, the LDL peak particle diameter, was defined by the estimated diameter or size of the major observed LDL subclass, to classify LDL subfraction pattern in a more continuous fashion (73). Single-spin DGUC procedures are designed for optimal resolution of prestained apo B containing lipoproteins (59,61,63,74,75). Depending on the used salt gradient and performed ultracentrifugation, up to 15 fractions can be isolated within the LDL density of 1.019 to 1.063 g/ml. A single-spin DGUC procedure using prestained whole plasma has been the basis of the research in our laboratory. This method reveals up to five different LDL subfractions. Quantification by densitometric scanning allows the calculation of a continuous variable K describing the relative contribution of each LDL peak height to the total LDL (76). In a comparison study, the number of LDL subfractions detected by GGE or DGUC was the same for more than 90% of the sera (77). However, different LDL subfractions were less well separated by GGE compared with DGUC. Furthermore, an advantage of the DGUC is that isolated subfraction samples are available for further biochemical analysis. In the near future, a capillary electrophoretic technique may combine rate, small amount of required plasma and distinguisability between different LDL subfraction (78).

Metabolic aspects of LDL subfractions

The intravascular formation of LDL subfractions involves the conversion of VLDL precursors (79,80), and possibly also a direct hepatic secretion of different IDL (81), or LDL subspecies (30,82). Previously, it was postulated that exchange of LDL cholesteryl ester for VLDL triglyceride, mediated by the cholesteryl ester transfer protein (CETP), results in a net transfer and a significant enrichment of the LDL with triglyceride (83-85). The subsequent action of lipoprotein lipase (LpL) or hepatic lipase (HtgL) results in hydrolysis of a significant amount of the LDL triglycerides and thereby a decrease in particle size (86-89). The rate and magnitude of exchange may depend upon the relative

pool size of triglyceride-rich lipoproteins versus the cholesteryl ester-rich lipoproteins. In general, this hypothesis of exaggerated triglyceride transfer and lipolysis can explain the predominance of small dense LDL in any form of hypertriglyceridemia. However, in FCH, the pool of triglyceride-rich lipoproteins is primairily enlarged and consists of chylomicron remnants as well (37). Furthermore, also VLDL heterogeneity may underly LDL heterogeneity. Large triglyceride-rich VLDL, resembling chylomicrons in patients with LpL deficiency, were found to be rapidly removed from the circulation (49). Several studies demonstrated that predominantly small VLDL particles secreted into the circulation are converted in LDL (48,90). Using stable isotopes, it was recently demonstrated that in subjects with predominantly dense LDL, both an increased production and reduced clearance of large VLDL occurred, which then undergo intravascular catabolism to successively smaller remnant particles, a pathway not apparent in subjects with larger more buoyant LDL (91). This and numerous other studies demonstrate the complexities of apo B particle metabolism. However, all these studies have in common that the metabolic actions of lipid transfer proteins and lipases, eventually combined with substrate specificity as well as heterogeneity among apo B precursor particles, could account for the multiple different LDL subfractions observed in normal and hyperlipidemic subjects.

Genetic aspects of LDL heterogeneity

Accumulating data suggest that the formation of LDL subfraction profiles is influenced genetically (92). Especially the finding of inherited LDL subfraction profiles in normolipidemic families (76,93) strongly suggests a genetic background. Initially, Fisher et al. reported a single genetic locus without dominance thought to be responsible for inheritance of LDL molecular weight quality in five families (94). Complex segregation analysis in healthy families (93) and in families of probands with familial combined hyperlipidemia (65) have indicated that LDL subclass pattern B, as assessed by gradient gel electrophoresis, is under the influence of a major gene or genes with a dominant or additive mode of inheritance. Two recent studies, a study in normolipidemic families (76) and a study in FCH families (66) from our laboratory, in which LDL subfractions were detected by DCUG confirmed a major gene effect. However, in contrast with the previous studies of Austin et al. (65,93), we observed a recessive mode of inheritance and gene frequencies significantly different between the normolipidemic and FCH population (66,76). However, all these studies including a recently performed heritability analysis of a continuous LDL peak particle diameter performed in twins (95), have indicated that genetic factors could account for at least 40% of the variation in LDL particle size and

density both in normolipidemic and hyperlipidemic subjects, with the remainder 60% due to nongenetic or environmental influences. Among these environmental factors are age, gender, body mass index, smoking habits, hormonal status in women (combined estimated effect of 20%), and lipid and lipoprotein levels (estimated effect of 40%) (66,76).

Additional genetic studies have linked candidate genes to the small dense LDL phenotype. Although only once reported, remarkably strong linkage (LOD score of 4.43) of pattern B to a gene locus near the LDL receptor on chromosome 19p was found in 51 family members of 9 probands with an 'atherogenic lipoprotein phenotype' (ALP), whereas weaker linkage was observed with the insulin receptor locus on same chromosome 19p (96). In another study, the apo B100 EcoRI polymorphism, previously associated with variation in plasma lipids, reportedly plays a role in the susceptibility to develop dense LDL in presence of visceral obese hyperinsulinemic men (97). Evidence for linkage of pattern B to three markers on the LDL receptor itself, the apo CIII gene on chromosome 11, the CETP gene on chromosome 16p and the manganese superoxide dismutase (MnSOD) gene on chromosome 6q has also been reported. However, no linkage was observed for other candidate loci tested: apo B, apo AI, apo (a), apo E/CI/CIII, LpL, and HDL binding protein (98,99). Although the investigated genetic loci have been identified by polymorphic DNA markers, which do not necessarily indicate the presence of causative mutations, it is remarkable that the protein products of genes with observed linkage have connections with metabolic pathways possibly involved in the generation or an impaired clearance of small dense LDL: (i) because small dense LDL particles have been shown to have reduced affinity for the LDL receptor (100-102), altered LDL receptor function or regulation could result in further impairment of plasma clearance of these LDL or their metabolic products. (ii) Apo CIII gene haplotypes are associated with variation in plasma triglyceride levels (51), which in turn could affect levels of small dense LDL. (iii) CETP may facilitate lipolytic conversion of larger to smaller LDL particles by promoting triglyceride transfer into the LDL core (103). (iv) A possible mechanism associated with MnSOD activity is unclear, but it is conceivable that defective function of MnSOD results in increased lipid hydroperoxides in plasma lipoproteins, with concomitant increase in oxidative susceptibility, or otherwise alter lipoproteins in a manner leading to formation of small dense more oxidizable LDL (70,99,104).

Although genetically influenced factors resulting in retardation of catabolism of triglyceride-rich lipoproteins or their remnants may have an etiological or contributory role to formation of small dense LDL, it is striking that mutations in the gene coding for LpL were not linked to the pattern B (99), even so, because the LpL Asn291→Ser was

recently found to be significantly linked to the presenting lipid phenotype in FCH families (50).

Altogether, LDL heterogeneity results from a variety of environmental influences and probably also from direct genetic factors. In a family one or more defects may be responsible for the major gene and additive effects identified by segregation analysis.

FCH AND PREMATURE ATHEROGENESIS

In spite of often mildly elevated lipid levels compared to other lipid disorders, a high prevalence of cardiovascular diseases occurs in FCH families. The explanation for premature cardiovascular disease in FCH may be attributed to the increased prevalence of small dense LDL (26,105).

One of the earliest events in the formation of atherosclerotic plaques is the massive accumulation of cholesterol in so called scavenger cells to convert into foam cells in the artery wall (106,107). Since normal receptor-mediated endocytosis of cholesterol via the LDL receptor initiates intracellular processes that prevent from further LDL uptake, alternative mechanisms are necessary to explain the foregoing intracellular cholesterol accumulation (108). Many lines of evidence support the hypothesis that oxidative modification of LDL plays a pivotal role in atherogenesis (109-112). However, this theory cannot be considered proven for the human disease (113). The oxidative modification hypothesis proposes that oxidative damage to LDL generates a series of modified forms of LDL (oxLDL) that are in a number of ways more atherogenic than native LDL. In contrast to native LDL, oxidized LDL is recognized and rapidly internalized by macrophage scavenger receptors (109), oxidized LDL (oxLDL) exhibits marked effects on the viability of endothelial cells and smooth muscle cells, and it alters the chemotactic activity of monocytes and monocyte-derived macrophages, all features of which have been implicated in atherogenesis (109). Special oxLDL receptors on the macrophages may not be down-regulated by the endocytosis of several forms of modified LDL and facilitate intracellular accumulation of oxLDL (114-117). Small dense LDL being more susceptible to oxidative modification (63,70,118) may increase the supply of oxLDL to these receptors. Recently, in subjects with FCH total LDL was found to be more prone to in vitro oxidation, due to the predominance of dense LDL particles. In addition, it was suggested that the decreased redox status of coenzyme Q10 in LDL from subjects with a dense LDL subfraction profile reflected the presence of already in vivo modified LDL due to lipid peroxidation in the circulation (119,120).

THERAPEUTIC OPTIONS IN FCH

Because of the up to 10-fold increased incidence of cardiovascular diseases in FCH patients (10,13), family screening and lipid-lowering treatment should be initiated. Both lowering of the total amount of atherogenic lipoproteins, i.e., LDL cholesterol and triglyceride-rich lipoproteins, as well as a reduction of the atherogeneity of LDL, i.e., reduction of total amount of small dense LDL particles should be aimed at. For this purpose, diet and lifestyle changes have usually insufficient effect, and consequently drug therapy is frequently indicated. Last decade, a spectrum of effective lipid-lowering drugs became available. The HMG-CoA reductase inhibitors are highly effective in patients with primary hypercholesterolemia to reduce LDL cholesterol (121). Although these drugs show some triglyceride reduction as well, less effect is observed in reduction of the amount of small dense LDL particles (18,122,123). Fibrates show a primary triglyceride-lowering effect (124). Convincingly related to this effect on triglyceride levels, a reduction of the amount of small dense LDL subfraction is observed, whereas the total amount of LDL cholesterol is unaffected or may even increase (18,125,126). Due to several side effects, nicotinic acid which reduces especially triglyceride levels (127) is less prescribed in Europe. Bile acid-binding resins are frequently contra-indicated in FCH because of an increase of VLDL concentration (128,129). The effectivity of antioxidants, i.e., vitamin E, C, β-carotene and flavonoids, to prevent LDL particles from oxidative modification is still under investigation. Although a reduction of in vitro LDL oxidizability has been observed (130-133) and a reduced risk of coronary heart disease was found (134-139), the results of these studies are not totally consistent.

A direct comparison of the HMG-CoA reductase inhibitor simvastatin and the fibrate gemfibrozil in the treatment of FCH subjects with a combined hyperlipidemic phenotype demonstrated the specific effect of both drugs. However, none of these agents alone completely normalized the lipid and lipoprotein profiles. Interestingly, an overal dense LDL subfraction profile, although less pronounced, remained despite a substantial triglyceride-lowering (18). This finding further supports the hypothesis of small dense LDL being present in FCH subjects irrespective of metabolic influences.

So far, the use of drugs should be based on which lipoprotein fraction is elevated the most, and probably a combination of a statin and a fibrate may be the therapy of choice in selected FCH patients with a high risk of cardiovascular disease. In the future, possibly more potent HMG-CoA reductase inhibitors, such as atorvastatin, which have also a strong triglyceride-lowering effect, may become the drug of choice (140,141).

CONCLUSION

Because of its large impact on total cardiovascular mortality, knowledge of the pathogenesis of the heterogenous FCH syndrome as well as the cause of the associated premature atherogenesis is essential. A major difficulty arises in identifying affected subjects, because a specific marker for the disorder is still lacking. Therefore, family investigation should be performed to verify the diagnosis in a patient with combined hyperlipidemia and/or premature cardiovascular disease.

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Chapter 3a

Effects of Gemfibrozil or Simvastatin on Apolipoprotein-B containing lipoproteins, Apolipoprotein-CIII and Lipoprotein(a) in Familial Combined Hyperlipidemia

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ABSTRACT

Background: Familial combined hyperlipidemia (FCH), characterized by elevated very low density lipoprotein (VLDL) and/or low density lipoprotein (LDL), is associated with an increased prevalence of premature cardiovascular disease. Therefore, lipid-lowering is frequently indicated. Material and methods: We evaluated in a double-blind, randomized fashion, parallel the effect of gemfibrozil (1200 mg/day) (n=40) or simvastatin (20 mg/day) (n=41) on lipids, apolipoprotein-B (apo-B) containing lipoproteins, apo-CIII and lipoprotein(a) (Lp(a)), in 81 well defined FCH patients. Results: While both drugs lowered plasma cholesterol- and triglyceride levels, gemfibrozil lowered plasma triglycerides more effectively by reduction of triglycerides in VLDL and LDL, whereas simvastatin was more effective in its reduction of total plasma cholesterol by exclusively decreasing LDL cholesterol. High density lipoprotein (HDL) increased to an equal extent on both therapies. Total serum apo-B levels were reduced with both drugs; however, gemfibrozil decreased apo-B only in VLDL+IDL, whereas simvastatin decreased apo-B in both VLDL+IDL and LDL. In keeping with a more effective reduction of VLDL particles, also a more pronounced reduction of apo-CIII was observed after gemfibrozil, which correlated with the reduction in plasma triglycerides. Baseline concentrations of Lp(a) showed a wide range in both treatment groups. Median Lp(a) levels increased after simvastatin, but were not affected with gemfibrozil. Conclusion: Both therapies exhibited their specific effects, although none of the drugs alone completely normalized the lipid profiles of these patients with FCH. Therefore, the choice of treatment should be based on the most elevated lipoprotein fraction, and in some cases, a combination of the two drugs may be indicated.

INTRODUCTION

Familial combined hyperlipidemia (FCH) is the most common of hereditary lipid disorders, with an estimated prevalence of 0.5-1.0% in the general population and charactarized by a strong predisposition for premature cardiovascular diseases in patients and first-degree relatives (1). Affected individuals have elevated very low density lipoprotein (VLDL) concentrations, low density lipoprotein (LDL) concentrations, or both. Furthermore, a low high density lipoprotein (HDL) cholesterol concentration, an elevated apolipoprotein-B (apo-B) concentration (2), and an increased prevalence of atherogenic small dense LDL subfractions is observed (3-5). Because of the absence of a specific clinical or metabolic marker for the disorder, and because of characteristic variability in the presenting lipid phenotype, family investigation is pivotal to establish the diagnosis FCH in a single patient (2). FCH seems to be a metabolically and genetically heterogenous lipid disorder (6), which, in general, is explained by an overproduction of VLDL particles (7,8). Furthermore, an impaired hydrolytic capacity of lipoprotein lipase (LpL) itself (9), or elevated plasma levels of a LpL inhibitor apo-CIII, possibly linked to genetic polymorphisms in the AI-CIII-AIV gencluster on chromosome 11 (10,11), may be related to the FCH phenotypes.

In general, elevated concentrations of lipoprotein(a) (Lp(a)) are related to an increased risk for cardiovascular disease, which is independent of the cardiovascular risk associated with elevated lipid levels (12). Although FCH is not associated with elevated Lp(a) levels (13), Lp(a) concentrations correlate positively with LpL mediated metabolism of triglyceride-rich lipoproteins (14). Reduction of the elevated pool of triglyceride-rich lipoproteins, as seen in FCH, may influence plasma Lp(a) levels (15).

Because of the high incidence of cardiovascular diseases in these patients, lipid-lowering treatment should be initiated. If diet and lifestyle changes have insufficient effect, drug therapy may be indicated. For this purpose, the HMG-CoA reductase inhibitor simvastatin, highly effective in patients with primary hypercholesterolemia (16), and fibrates like gemfibrozil with a primary triglyceride-lowering effect (17) are useful.

Until now few comparative data are available about the different effects of these agents in FCH. In the present study we describe the baseline levels of lipids, apo-B containing lipoproteins, apo-CIII and Lp(a) in well defined patients with FCH, and compare the results of treatment with either gemfibrozil or simvastatin on these parameters.

MATERIAL AND METHODS

Subjects

In total, 81 FCH probands from 3 centers participated in this study. A subgroup of this population has been studied regarding LDL subfraction profiles and LDL oxidizability (5). In the present study the effects of treatment with either gemfibrozil or simvastatin on serum lipids, lipoproteins, apolipoprotein CIII and Lp(a) in all 81 FCH probands are presented.

At the end of the screening period, all FCH patients met the following criteria: (i) a total serum cholesterol >6.5 mmol/l and triglyceride level between 2.3 and 5 6 mmol/l, without hypolipidemic drugs and on a standard-lipid lowering diet (30 energy% fat, P/S ratio of 2:1, and a cholesterol intake <300 mg/day), (ii) at least 1 first-degree relative with significant hypercholesterolemia, hypertriglyceridemia or both, (iii) a positive family history of premature coronary heart disease, (iv) total apo-B levels above 1200 mg/l. Furthermore, all patients were >30 years old and patients with secondary causes for dyslipidemia or with apolipoprotein phenotype E 2/2 were excluded The study protocol was approved by the local medical ethical committees, and all patients participated after informed consent was obtained

Experimental design

This study was a double-blind trial with a double dummy design, divided into a screening period (weeks -8 to -5), a baseline placebo period (weeks -4 to day 0) and an active treatment period (day 0 to week 12) In the screening period, patients were taken off all hypolipidemic drugs, receiving a standard lipid-lowering diet When total plasma cholesterol concentration exceeded 6 5 mmol/l, and plasma triglyceride concentration remained between 2 3 and 5 6 mmol/l, the patients entered the baseline placebo period of the study. During this period, each patient received 2 bottles, one containing placebo matching gemfibrozil and one containing placebo matching simvastatin During the active treatment period, patients were randomly assigned to receive either simvastatin 20 mg/day together with a placebo matching gemfibrozil (n=41) or gemfibrozil 1200 mg/day together with a placebo matching simvastatin (n=40) In the present study we compared concentrations of total plasma cholesterol and plasma triglycerides obtained at the end of the entering period, at the end of the placebo period and at two time points (6 and 12 weeks) during the period of active treatment. Furthermore, analyses of lipoproteins, apoproteins and Lp(a) at the end of the placebo-controlled period were compared with the same data at the end of the active treatment period

Plasma

Blood samples were obtained after an overnight fast and collected into ethylenediaminetetraacetic acid (EDTA) (1mg/ml)-containing vacutainers. Plasma was isolated immediately and a saccharose solution (600 mg/ml H_2O) was added to prevent denaturation of VLDL and LDL during freezing; samples were stored at -80 °C for 4 to 15 weeks. The analysis of apo-CIII and Lp(a) was performed in the lipid research laboratory, university hospital of Utrecht, Apo-B was determined in the lipid research laboratory, university hospital of Amsterdam, all other lipid determinations were performed in the lipid research laboratory, university hospital of Nijmegen.

Analytic methods

Very low density lipoproteins (VLDL) and intermediate density lipoproteins (IDL) were isolated together by ultracentrifugation at density ≤ 1.019 g/ml for 16 hours at 40,000 rpm in a fixed angle rotor (TFT 45.6 rotor, Kontron, Zürich), in a Beckman L7-55 ultracentrifuge (Beckman, Palo Alto, USA) (18). After removal of VLDL+IDL, we measured cholesterol and triglycerides in the remaining plasma and in total plasma. HDL was determined from whole plasma by the polyethylene glycol method (19). All cholesterol and triglyceride measurements were determined by enzymatic methods (BoerhringerMannheim, Mannheim, Germany, cat. no. 237574 and Sera Pak, Tournai, Belgium cat. no. 6669, respectively) with a centrifugal analyzer (Multistat III; Instrumentation Laboratory, Lexington, MA). VLDL+IDL cholesterol and triglyceride were calculated by subtraction. Apo-B and apo-AI concentrations in total plasma and apo-B concentrations in the fraction that remained after VLDL+IDL removal, were determined by nephelometry (20). VLDL+IDL-apo-B was calculated by subtraction. Apo-CIII was determined quantitatively by radial immunodiffusion using plates and apo-CIII standards, according to manufacturer's instructions (Daiichi Pure Chemicals, Ltd, Japan). The diameter of the precipitation ring was measured by an investigator unaware of the specimen's identity. Serial samples from each subject were measured in the same assay. Lp(a) concentrations were determined by measuring the apoprotein(a) moiety in a commercially available solid phase two-site immunoradiometric assay (IRMA) using 2 different specific anti-apoprotein(a) monoclonal antibodies (Pharmacia, Uppsala, Sweden).

Statistics

Results are expressed as mean $\pm$ SD and as median for Lp(a). Statistical analysis of alterations within one group of treatment was performed with Wilcoxons' Signed Rank

test Differences in terms of percentage between the two groups of treatment were analyzed with the Mann-Whitney test. A two-tailed probability value of less than 0 05 was considered to be significant. The statistical analysis were performed with procedures available in the SPSS PC+ (Statistical Package for the Social Sciences) software package Version 4.0 (SPSS Inc. Chicago, Il, USA).

RESULTS:

Participants

After the screening period (4 weeks), participants who exhibited a IIb phenotype (total plasma cholesterol concentration $\geq$6.5 mmol/l and plasma triglyceride concentration $\geq$2.3 mmol/l) were allowed to enter the placebo period. Due to variability in presenting lipid phenotype during the placebo period (4 weeks), 55 patients exhibited a phenotype IIb, 12 patients exhibited a phenotype IIa (total plasma cholesterol concentration $\geq$6.5 and plasma triglyceride concentrations $<$2 3 mmol/l), and 14 patients exhibited a phenotype IV (total plasma cholesterol concentration $<$6 5 mmol/l and plasma triglyceride concentration $\geq$2 3 mmol/l, respectively) at the end of the baseline placebo period. Description of the two treatment groups, the body mass index (BMI), and the blood pressure are presented in Table 1.

Table 1: Age, gender,bloodpressure and body mass index of 81 patients with Familial Combined Hyperlipidemia

	gemfibrozil	simvastatin
n	40	41
Age (years)	53 4 ± 9 4	50 4 ± 10 8
Gender M/F	26/14	32/9
Systolic bloodpressure (mmHg)	131 ± 18	127 ± 13
Diastolic bloodpressure (mmHg)	84 ± 9	81 ± 8
Body mass index (kg/m^2)	27 2 ± 3 0	26 6 ± 2 7

Values are presented as mean ± SD

Effect of treatment on lipid and lipoprotein concentrations

In Figure 1 the changes of the mean total plasma cholesterol and plasma triglyceride concentrations during the placebo period and the period of treatment are presented.

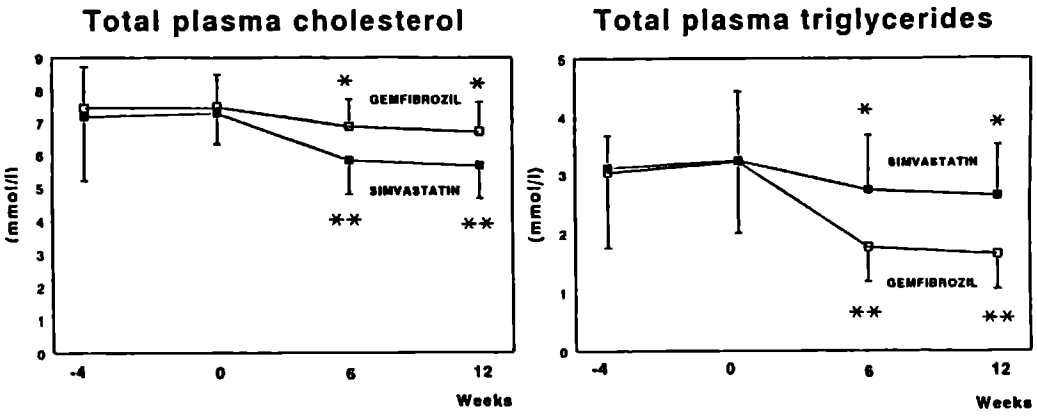

Figure 1: Change in plasma concentrations (mmol/l) of total cholesterol (TC) and triglycerides (TG) during the placebo period (week -4 to day 0), and on two time points (week 6 and week 12) during the period of active treatment with either gemfibrozil (□), (n=40) or simvastatin (■), (n=41) of patients with familial combined hyperlipidemia; *p<0.001, ** p<0.0001, compared with the baseline level (week 0).

At entry and at the end of the placebo period there were no significant differences in mean lipid and lipoprotein concentrations between the two groups, and as shown in Figure 1, mean lipid levels were unaffected during the placebo period. Lipoprotein levels at the end of the baseline period and after therapy are summarized in Table 2. During the treatment period, the BMI appeared to be constant in both groups. Gemfibrozil affected total triglyceride levels in plasma as well as in the VLDL+IDL and LDL fraction significantly more than simvastatin, whereas simvastatin induced the largest reduction in total plasma cholesterol with an exclusive reduction of LDL-cholesterol. Gemfibrozil tended to increase LDL-cholesterol (+8.3%, not significant). In both groups, the reduction of cholesterol and triglycerides in the VLDL+IDL fraction was responsible for the major decrease of total plasma cholesterol and triglycerides. With both drugs, HDL-cholesterol concentrations increased significantly to an equal extent.

Effect of treatment on apo-B containing lipoproteins

Alterations in the apo-B concentrations in total plasma, the VLDL+IDL fraction and the LDL fraction are presented in Figure 2. Simvastatin and gemfibrozil reduced total plasma apo-B (-25.6% and -13.7%, respectively, but simvastatin was more effectively if measured in percentage of change, p=0.02). Simvastatin reduced apo-B in both the

VLDL+IDL and the LDL fraction (-35.4% and 16.7%, respectively).

Table 2: Lipid and lipoprotein concentrations at the end of the placebo period (=before) and after treatment (=after) with either gemfibrozil (n=40) or simvastatin (n=41) of patients with Familial Combined Hyperlipidemia

	Drug	Before	After	Delta (%)	p
TC	G	7.50 ± 0.98	6.73 ± 0.91	-9.5 ± 13.2	0.0001
	S	7.30 ± 0.96	5.67 ± 1.00	-22.4 ± 9.2**	<0.0001
TG	G	3.22 ± 1.22	1.64 ± 0.59	-45.5 ± 19.7	<0.0001
	S	3.20 ± 1.20	2.63 ± 0.88	-14.6 ± 27.8**	0.0003
HDL-C	G	0.91 ± 0.20	1.07 ± 0.26	17.4 ± 17.6	<0.0001
	S	0.84 ± 0.19	0.95 ± 0.21	13 9 ± 13 1	<0.0001
VLDL+IDL-C	G	2.43 ± 0.84	1.15 ± 0.84	-50.6 ± 20.6	<0.0001
	S	2.60 ± 1.02	1.56 ± 0.62	-37.6 ± 20.1**	<0.0001
VLDL+IDL-TG	G	2.61 ± 1.18	1.43 ± 1.15	-44.7 ± 28.0	<0.0001
	S	2.59 ± 1.04	2.08 ± 0.79	-14.1 ± 32 6**	0.0008
LDL-C	G	4.08 ± 0.99	4.29 ± 1.26	8.3 ± 41 9	NS
	S	3.87 ± 0.98	3.16 ± 0 87	-16.3 ± 21.0**	<0 0001
LDL-TG	G	0.67 ± 0.20	0.49 ± 0.13	-22.9 ± 19.9	<0.0001
	S	0.60 ± 0.12	0.53 ± 0.12	-10.9 ± 16.8*	0.0003

Values are presented in mmol/l as mean ± SD; TC, total plasma cholesterol; TG, plasma triglycerides; VLDL, very low density lipoproteins; IDL, intermediate density lipoproteins; HDL, high density lipoproteins; LDL, low density lipoproteins; G gemfibrozil, S simvastatin; NS, not significant; * $p<0.001$, ** $p<0.0001$, S versus G.

Gemfibrozil, on the contrary, reduced apo-B only in the VLDL+IDL fraction (-42.4%; difference simvastatin vs. gemfibrozil in terms of percentage change, $p<0.0001$), without affecting the LDL fraction (+5.5%, not significant; difference simvastatin vs. gemfibrozil in terms of percentage $p<0.0001$).

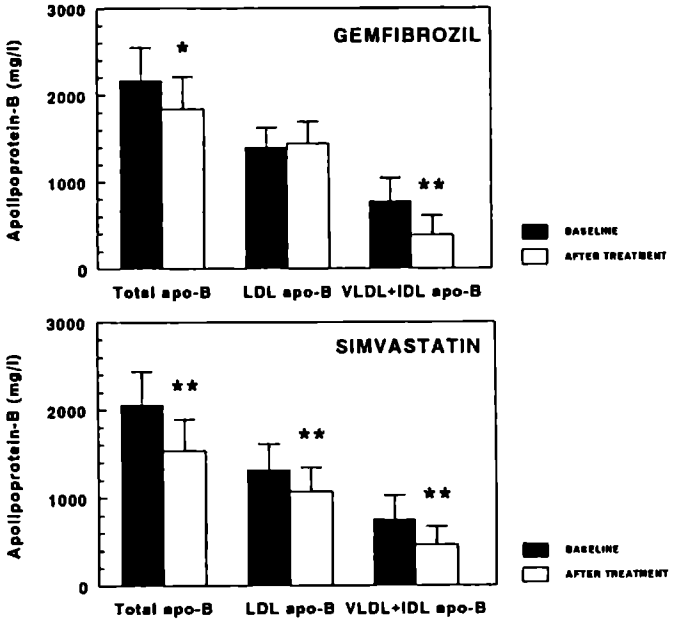

Figure 2: Effect of treatment with either gemfibrozil (n=40) or simvastatin (n=41) on apolipoprotein-B containing lipoproteins in total plasma, the LDL fraction and the VLDL+IDL fraction of patients with familial combined hyperlipidemia, black before and hatched after treatment; * $p=0.0001$, ** $p<0.0001$.

Effect of treatment on apo-AI and -CIII

Changes in apoprotein-AI and -CIII are summarized in Table 3. The increase in HDL-cholesterol on both treatments was not accompanied by an increase of apo-AI in both groups. Apo-CIII decreased significantly with both therapies, but more pronounced after gemfibrozil. In general, the absolute decrease in Apo-CIII concentrations coincided with the decrease of VLDL+IDL particles, as measured by apo-B concentrations in this fraction. There was a significant correlation between the changes in apo-CIII and the change in plasma triglycerides after gemfibrozil and simvastatin ($r=0.69$ and $r=0.67$;

p=0.001, respectively).

Table 3: Lipoprotein (a), apolipoprotein-AI and apolipoprotein-CIII at the end of the placebo period (=before) and after treatment (=after) with either gemfibrozil (n=40) or simvastatin (n=41) of patients with Familial Combined Hyperlipidemia

	Drug	Before	After	Delta (%)	P
Lp(a)	G	18.0	20.7	10.2 ± 38.9	NS
	S	19.6	26.1	14.9 ± 31.1	0.015
Apo-A I	G	131.5 ± 24.7	134.4 ± 24.1	2.9 ± 12.2	NS
	S	136.7 ± 25.4	133.7 ± 22.2	-0.1 ± 18.1	NS
Apo-CIII	G	14.8 ± 3.4	10.6 ± 2.3	-26.0 ± 17.2	<0.0001
	S	14.7 ± 4.7	13.6 ± 3.6	-6.4 ± 20.3*	0 005

Values are presented in mg/dl as median for Lp(a) [lipoprotein(a)] and as mean ± SD for apo-AI (apolipoprotein-AI) and apo-CIII (apolipoprotein-CIII); S, simvastatin; G, gemfibrozil; NS, not significant; *p<0.0001, S versus G.

Effect of treatment on Lp(a)

Before and after therapy Lp(a) values showed a wide distribution. Effects on median levels are summarized in Table 3. The effect of gemfibrozil was not significant, 18 out of 40 patients showed a decrease, but conversely, 27 out of 41 patients showed an increase of Lp(a) levels on simvastatin.

DISCUSSION

In spite of often mildly elevated lipid levels compared to other lipid disorders, there is a high prevalence of cardiovascular diseases in FCH families. Consequently, family screening and pharmacological intervention in affected individuals may be indicated (21). The increased tendency to premature cardiovascular disease in these patients must be related to the increased pool of triglyceride-rich apo-B containing lipoproteins in conjunction with elevated LDL cholesterol levels (22,23). In addition, hypertriglyceridemia, as found in FCH with type IIb and IV phenotypes, is associated

with augmented exchange of lipids between VLDL, HDL and LDL, mediated by cholesteryl transfer protein (CETP), which may result in more small dense atherogenic LDL particles in total LDL (24,25). Elevated apo-CIII concentrations may be associated with hypertriglyceridemia, because of a possible inhibition of the LpL activity by apo-CIII (26). Furthermore, decreased HDL cholesterol, apo-AI concentrations and elevated concentrations of Lp(a), may also be involved in the atherogenesis in FCH (13,27).

Recently, large prevention trials and studies with solid cardiovascular end-points have established now that cholesterol lowering results in a decrease in mortality from cardiovascular disease, and regression of atherosclerotic lesions (28-31). Although the study populations did not met the FCH diagnostic criteria, it can be deduced from these results that lipid lowering may affect mortality and morbidity from cardiovascular events in FCH patients as well, because lipid parameters associated with an increase in cardiovascular risk are also influenced in the present study.

The observed reduction of total plasma cholesterol and plasma triglyceride concentrations with simvastatin (-22.4 % and -9.5 %, respectively) and gemfibrozil (-14.6 % and -45.5 %, respectively) after 12 weeks of treatment in our study are in accordance with previous reports (32,33). To investigate the effect of both therapies on triglyceride-rich apo-B containing lipoproteins, VLDL and IDL particles were isolated together. This explains the relatively large contribution of VLDL+IDL cholesterol and the relatively small contribution of LDL cholesterol to total plasma cholesterol, both before and after treatment. The increase of HDL cholesterol with gemfibrozil therapy agrees with previous studies as well (32,34), but the increase of HDL on simvastatin, despite a less pronounced reduction of triglycerides, is larger than previously reported (32). The mean apo-AI concentration was unaffected, despite an increase in HDL cholesterol and may reflect HDL cholesterol particles containing less protein (35). The effects on LDL cholesterol are comparable with other reports (32,33). The depletion of triglycerides in the VLDL+IDL fraction, leading to more small dense particles, which are more likely to be converted into LDL particles is suggested to be a cause of the observed increase of LDL cholesterol after gemfibrozil (36). On the other hand, a triglyceride reduction in the LDL particle, more pronounced after gemfibrozil, yields more buoyant LDL subfractions, as we have demonstrated before (5,37). These buoyant LDL subfractions may be less atherogenic, because an increased resistance against oxidative modification has been described (18). We observed that simvastatin most effectively reduced the total LDL cholesterol concentration, but did not affect the LDL subfraction profile of FCH patients (5), which was recently demonstrated for pravastatin as well (38).

All patients had moderate to severe elevations of apolipoprotein-B levels. Both therapies reduced total apo-B, but in accordance with the reduction of total apo-B containing particles, simvastatin reduced apo-B in both the VLDL+IDL and LDL fraction more effectively than gemfibrozil, which only reduced VLDL+IDL-apo-B. Since VLDL+IDL and LDL particles contain only one molecule of apo-B per particle, a reduction of apo-B observed in these fractions reflects a reduction of the number of potential atherogenic particles per isolated fraction.

It has been suggested that an elevated apo-CIII concentration may be a metabolic marker for FCH. The decrease of the apo-CIII concentrations in this study, however, significantly correlated with the decrease in triglyceride-rich lipoproteins and parallelled the reduction of the particle numbers in the VLDL+IDL fraction. Recently, Patsch et al, found a lower apo-CII/apo-CIII ratio, due to an elevated apo-CIII concentration, in VLDL particles of patients with type IIb hyperlipidemia, which was related to sequence variations in the minor allel of the AI/CIII/AIV gene cluster on chromosome 11 (39). From our results, it remains to be seen whether increased apo-CIII concentrations have some role in the pathophysiology of FCH and can serve as a metabolic marker.

Sofar, the effects of hypolipidemic drugs on Lp(a) levels have not been very succesful. Studies with simvastatin demonstrated either no effect (40), or an increase of Lp(a) concentrations (15). Although it has been shown that Lp(a) may be bound to triglyceride-rich lipoproteins (41) and that LpL mediated clearance of triglyceride-rich lipoproteins influence Lp(a) concentrations (14), the mechanism behind this association remains unclear. Synthesis of Lp(a) in the liver may share some characteristics with VLDL synthesis, which is dependent on the amount of free fatty acids to the liver cell (42). It is still unclear which causes underly this possible increased supply of free fatty acids to the liver cell in FCH. Gemfibrozil, however, which may have some effect on the release of fatty acids from the adipose tissue, had no significant effect on Lp(a) concentrations.

In conclusion, we directly compared the effects of two drugs with different working mechanisms in a well-defined large group of FCH patients, in order to support the choice of treatment for this heterogeneous lipid disorder. None of these agents completely normalized the lipid and lipoprotein profiles. However, each agent has its specific effect in the treatment of subjects with both elevated total plasma cholesterol and triglyceride concentrations. The use of drugs should be based on which lipoprotein fraction is elevated the most during several visits to the out-patient clinic, and probably a combination of gemfibrozil and simvastatin may be the therapy of choice in selected FCH patients with a

high risk of cardiovascular disease.

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Chapter 3b

Comparison of Gemfibrozil Versus Simvastatin in Familial Combined Hyperlipidemia and Effects on Apolipoprotein-B-Containing Lipoproteins, Low-Density Lipoprotein Subfraction Profile and Low Density Lipoprotein Oxidizability

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ABSTRACT

We evaluated in a double-blind, placebo-controlled randomized trial of 45 well defined patients with familial combined hyperlipidemia, the effect of gemfibrozil (1,200 mg/day) or simvastatin (20 mg/day) on apolipoprotein-B (apo-B) containing lipoproteins, low-density lipoprotein (LDL) subfraction profile and LDL-oxidizability. Although both therapies lowered plasma cholesterol and triglycerides concentrations, gemfibrozil reduced plasma triglycerides more effectively and simvastatin reduced plasma cholesterol more effectively. LDL cholesterol was reduced by simvastatin. With both drugs, total serum apo-B concentration decreased. With gemfibrozil, this was due to an exclusive reduction (-46%) of very low/intermediate-density lipoprotein (VLDL+IDL) apo-B, whereas simvastatin decreased apo-B in both VLDL+IDL and LDL (34% and 15%, respectively). Initially, a dense LDL subfraction profile was present in all patients. The decrease of LDL cholesterol with simvastatin was due to a decrease in all isolated LDL subfractions except LDL2; gemfibrozil increased LDL1 cholesterol and LDL2 cholesterol ($p=0.001$) and reduced LDL4 cholesterol, resulting in a more buoyant LDL subfraction profile compared with simvastatin. In both groups, a predominance of small dense LDL remained despite therapy. LDL fatty acid composition showed a shift from oleic acid to linoleic acid after gemfibrozil; arachidonic acid increased after simvastatin. Vitamin E was lower after gemfibrozil. In the measurements of LDL oxidation, only the oxidation rate was significantly reduced with simvastatin. Thus, quantitative and qualitative changes of LDL cholesterol had only a small effect on total in vitro LDL oxidizability in this population suffering from familial combined hyperlipidemia.

INTRODUCTION

Familial combined hyperlipidemia is a metabolically and genetically heterogeneous lipid disorder (1-3) with affected individuals exhibiting elevations of total cholesterol, triglyceride, or both, at least in partially caused by very-low-density lipoprotein (VLDL)-apo-B (apo-B) overproduction (4). Its prevalence is estimated to be 0.5% to 1.0% (2) and the trait predisposes to premature cardiovascular complications (1,5). Because of the absence of a specific metabolic parameter characteristic of familial combined hyperlipidemia, family studies are pivotal for diagnosis. The current diagnosis is based on the following criteria (1,6): prevalence of multiple lipoprotein phenotypes in first-degree relatives, premature atherosclerosis, decreased high density lipoprotein (HDL) cholesterol levels, elevated plasma levels of apo-B (7), impaired clearance of VLDL-remnants (8) and an increased prevalence of small dense low-density lipoprotein (LDL) (7). A predominance of small LDL and its enhanced susceptibility to copper mediated oxidative modification (9) is associated with atherogenesis (10,11). Because of the reported increased risk for premature atherosclerosis, treatment with lipid-lowering drugs is frequently indicated (12,13). In this report we described the baseline lipoprotein concentrations, LDL subfraction profiles and LDL oxidizability of well-defined patients with familial combined hyperlipidemia and compared the effectiveness of treatment with either gemfibrozil or simvastatin on these parameters in a double-blind, placebo-controlled fashion.

METHODS

subjects

In all, 81 out-patients suffering from familial combined hyperlipidemia were selected by 3 participating centers, to evaluate the effect of treatment with either gemfibrozil or simvastatin on serum lipids. Fourty-five patients of the 3 centers were randomly assigned for more extensive biochemical studies. They participated after informed consent was obtained. At entry into the placebo baseline period, patients met the following inclusion criteria: total serum cholesterol >6.5 mmol/l and triglyceride level between 2.3 and 5.6 mmol/l; at least 1 first-degree relative with significant hypercholesterolemia, hypertriglyceridemia or both; a positive family history of premature coronary heart disease; total apo-B 100 levels $>1,200$ mg/dl, and age >30 years. Patients with secondary causes for dyslipidemia or with apolipoprotein phenotype E 2/2 were excluded.

Study design

This study was a double-blind trial with a double dummy design, divided into three consecutive periods over 20 weeks. During the first period (weeks -8 to -5) selected patients who had taken no lipid-lowering drugs for ≥4 weeks received a standard lipid-lowering diet. The second period (week -4 to day 0) was a baseline placebo period. Each patient received 2 bottles, 1 containing placebo matching gemfibrozil and 1 containing placebo matching simvastatin. During the third period of active treatment (day 0 to week 12) patients were randomly assigned to receive either simvastatin 20 mg/day together with placebo matching gemfibrozil (n=23) or gemfibrozil 1,200 mg/day together with placebo matching simvastatin (n=22). In the present study data obtained at the end of the placebo period (day 0) were compared with results obtained at the end of the period of active treatment (day 84).

Plasma

Blood samples were obtained after an overnight fast and collected into vacutainers containing 1 mg/ml of ethylenediaminetetraacetic acid. Plasma was isolated immediately and a saccharose solution (600 mg/ml H_2O) was added to prevent denaturation of LDL during freezing; samples were stored at -80 °C for 4 to 15 weeks. All determinations were performed in the lipid research laboratory of the university hospital Nijmegen.

Analytic methods

VLDL+intermediate-density (IDL) (density [d]≤1.019 g/ml) were isolated by ultracentrifugation for 16 hours at 40,000 rpm in a fixed angle rotor (TFT 45.6 rotor, Kontron, Zürich), in a Beckman L7-55 ultracentrifuge (Beckman, Palo Alto, California) (14). After removal of VLDL+IDL, cholesterol and triglycerides were measured in the remaining plasma and in total plasma. HDL was isolated from whole plasma by the polyethylene glycol method (15). All cholesterol and triglyceride measurements were determined by enzymatic methods (Boerhringer-Mannheim, Mannheim, Germany, [catalog. no. 237574] and Sera Pak, Miles, Tournay, Belgium [catalog. no. 6669], respectively) with a centrifugal analyzer (Multistat III, Instrumentation Laboratory, Lexington, Massachusetts). VLDL+IDL cholesterol and triglyceride were calculated by subtraction. Apo-B concentrations in total plasma and in fractions that remained after VLDL+IDL removal, were determined by nephelometry (16). For improving the accuracy of the results, the values obtained in our laboratory were recalculated on the basis of values determined by an radioimmunoassay, standardized by the International

Union of Immunological Societies, in 40 specially selected fresh frozen control sera provided by Dr. S. Marcovina (Northwest Lipid Research Laboratory, Seattle Washington). VLDL+IDL Apo-B was calculated by subtraction.

Low density lipoprotein subfractionation

LDL subfraction analysis before and after treatment was performed by density gradient ultracentrifugation (14). For each patient at both occasions, this analysis was carried out in the same run. After ultracentrifugation the LDL subfractions were visible as distinct bands in the middle of the tube. Up to 5 LDL subfractions could be distinguished concentrated in the following density ranges: LDL1 (1.030-1.033g/ml), LDL2 (1.033-1.040 g/ml), LDL3 (1.040-1.045 g/ml), LDL4 (1.045-1.049 g/ml), and LDL5 (1.049-1.054 g/ml). The ultracentrifugation tubes, containing the LDL subfractions stained with Coomassie Brilliant Blue R, were placed in a specially designed rack and photographed. Accurate documentation of the different LDL subfraction patterns was obtained by scanning the obtained slides in triplicate on a LKB 2202 ultrascan laser densitometer (Pharmacia LKB, Uppsala, Sweden). The mean relative peak heights (h1 to h5) of the LDL subfractions (LDL1 to LDL5) on the 3 scans were used to calculate parameter K as a continuous variable, to describe each individual LDL subfraction pattern (17). When LDL4 and/or LDL5 were detected their relative peak heights were included in the formula LDL[100%] = LDL1[%h1] + LDL2[%h2] + LDL3[%h3] by converting %h3 into %h3' in which %h3'= (%h3 + %h4 + %h5). Variable K was calculated by: K = (%h1-%h3')/(%h2-%h1+1) (17). A negative value ($-1 < K < 0$) reflects a dense subfraction profile, an intemediate subfraction profile is described by $K=0$, and a complete buoyant profile delivers a positive K value ($0 < K < 1$). After photography, the LDL subfractions were accurately isolated by aspiration with a rubber bulb pipet and total cholesterol content of each subfraction was determined.

Determination of fatty acids and vitamin E in low-density lipoprotein

Analysis of fatty acids, extracted from LDL by vortex mixing with 3 ml n-hexane, was performed by gas chromatography (Varian 3400 GC, Houten, The Netherlands) (18). Vitamin E concentrations were determined by high-performance liquid chromatography (HPLC Spectra Physics Model 8800), with fluorescence detection (14). For extraction of vitamin E, 0.2 ml LDL was vortex mixed with 2 ml acetone and 2 ml petroleum ether.

Oxidation of low-density lipoproteins

The oxidation experiments were performed as described by Esterbauer et al (19), with modifications by Kleinveld et al (20).

Statistics

Results are expressed as mean ± SD. Statistical analysis of alterations within one group of treatment was performed with Wilcoxon's Signed Rank test. Differences in percentage between the 2 groups of treatment were analyzed with Wilcoxon's Rank Sum test. A 2-tailed probability value <0.05 was considered significant. The statistical analysis were performed with procedures available in the SPSS PC+ (Statistical Package for the Social Sciences) software package version 3.1.

RESULTS

Population

At the entry to the placebo period, all 45 patients described in this study met the inclusion criteria. Therefore, all patients had hyperlipidemia phenotype IIB. The gemfibrozil group (5 women and 17 men) and the simvastatin group (5 women and 18 men) were similar in mean age and body mass index (mean age 53.9 ± 9.8 vs. 53.1 ± 10.3 years, respectively; body mass index 27.4 ± 3.1 vs. 26.6 ± 2.9 kg/m^2, respectively). The lipid and lipoprotein levels, body mass index and age of the 45 patients were equal compared with the initial population.

The effect of treatment on plasma lipid and lipoprotein levels

The lipid and lipoprotein levels of the patients with Familial Combined Hyperlipidemia before and after treatment with gemfibrozil (n=22) or simvastatin (n=23) are summarized in Table 1. There were no significant differences of lipid concentrations between the two groups at baseline. Gemfibrozil significantly affected total triglyceride levels in plasma as well as in the VLDL+IDL fraction, whereas simvastatin induced the largest reduction in total plasma cholesterol. On the other hand, VLDL+IDL cholesterol was reduced with both therapies to the same extent. The largest contribution to the reduction in total cholesterol and triglycerides was generated by a decrease in the VLDL+IDL fraction. LDL cholesterol was only reduced by simvastatin and even tended to increase after gemfibrozil therapy. HDL cholesterol levels increased with both drugs.

Table 1: Changes in Lipids and Lipoproteins in Subjects With Familial Combined Hyperlipidemia After Treatment With Gemfibrozil or Simvastatin

	Medication	Before	After	Change (%)	P
Total cholesterol	G	7.54 ± 1.13	6.51 ± 0.91	-12.9 ± 11.7	<0.001
	S	7.15 ± 0.87	5.58 ± 1.12	-22.2 ± 9.4*	<0.001
Triglycerides	G	2.90 ± 0.91	1.42 ± 0.41	-48.1 ± 18.0	<0.001
	S	3.27 ± 1.19	2.61 ± 0.86	-15.9 ± 25.8†	0.003
VLDL+IDL triglycerides	G	2.24 ± 0.77	0.96 ± 0.38	-54.5 ± 20.0	<0.001
	S	2.58 ± 1.02	2.04 ± 0.74	-15.1 ± 24.2†	0.005
VLDL+IDL cholesterol	G	2.26 ± 0.76	0.99 ± 0.47	-55.3 ± 30.3	<0.001
	S	2.62 ± 0.90	1.50 ± 0.51	-40.9 ± 18.1*	<0.001
LDL-C	G	4.36 ± 0.86	4.59 ± 0.92	+9.3 ± 36.6	NS
	S	3 68 ± 1.01	3.12 ± 0.98	-13.3 ± 21.6*	<0.001
HDL-C	G	0.92 ± 0.18	1.06 ± 0.26	+15.3 ± 20.9	0.003
	S	0.82 ± 0.21	0.94 ± 0.21	+16.3 ± 13.1	<0.001
Apo-B	G	213 6 ± 41.0	164.4 ± 38.8	-21.1 ± 20.7	<0.001
	S	202.9 ± 41.3	152.2 ± 40 6	-25.2 ± 9.9	<0.001

Values are presented in mmol/l as mean ± SD, except apolipoprotein-B (apo-B) which is presented in mg/dl; G indicates gemfibrozil (n=22); S, simvastatin (n=23); * P<0.01, † P<0.001, G versus S. VLDL indicates very low density lipoprotein; IDL, intermediate density lipoprotein; HDL, high density lipoprotein; LDL, low density lipoprotein.

Effect of treatment on apolipoprotein-B lipoproteins

A significant correlation was found between apo-B and total apo-B related cholesterol (total cholesterol minus HDL cholesterol) (Pearsons' correlation coefficient 0.91; p=0.001) (Figure 1). The concentrations of VLDL+IDL cholesterol and LDL cholesterol also correlated with their related apo-B content (correlation coefficient 0.70; p=0.001; 0.63; p=0.001, respectively). Both therapies reduced total apo-B to a similar

extent. Gemfibrozil reduced apo-B only in the VLDL+IDL fraction, while simvastatin reduced apo-B in the VLDL+IDL and in the LDL fraction (Figure 2).

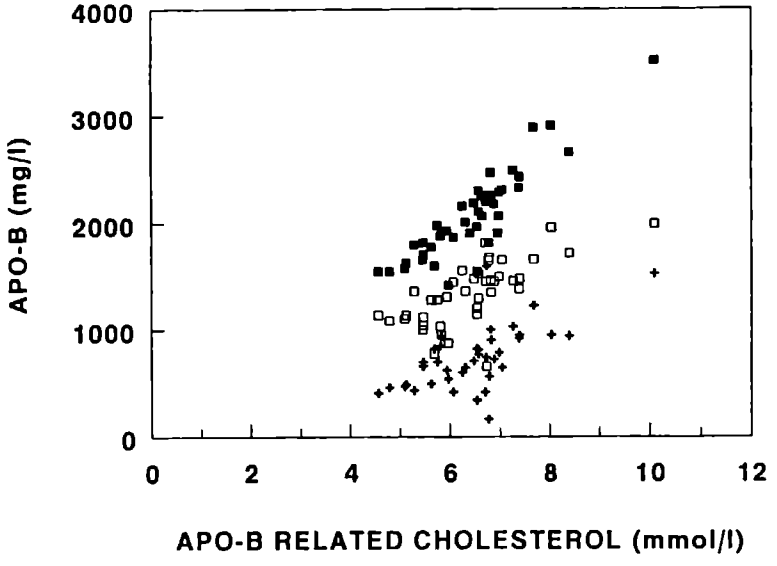

Figure 1: Correlation between total apo-B related cholesterol (total plasma cholesterol minus HDL cholesterol) in mmol/l and total plasma apo-B (■), VLDL+IDL apo-B (+) and LDL apo-B (□) (in mg/l) in 45 patients with FCH.

Effect of therapy on LDL subfraction profile and K value

Initially in all patients, LDL tended to be monodisperse, consisting of a limited number of LDL subfractions, with a predominance of intermediate dense (LDL2) and small dense (LDL3 and LDL4) subfractions. In 3 of 45 LDL subfraction profiles a clear very dense LDL5 band could be distinguished, which in all cases completely disappeared after treatment (1 after gemfibrozil and 2 after simvastatin). This sporadic LDL5 appeared in 3 patients with lipoprotein levels comparable to the other subjects, was excluded from further statistical analysis. Gemfibrozil treatment induced a less dense LDL subfraction profile, without a reduction of total LDL cholesterol, consisting of LDL1 to LDL4, with a

predominance of LDL1 to LDL3 as the main LDL subfractions (Figure 3). The ratio cholesterol/triglyceride within the LDL particle increased, whereas that of triglyceride/apo-B decreased, probably due to a reduction of triglyceride per LDL particle. The ratio cholesterol/apo-B did not change (Table 2). Simvastatin treatment reduced total LDL, but did not induce a major shift to a less dense LDL subfraction profile as seen with gemfibrozil. The amount of cholesterol in all LDL subfractions, except LDL2, was significantly reduced (Figure 3). Neither the ratio cholesterol/triglyceride, nor that of the ratio cholesterol/apo-B, or triglyceride/apo-B changed significantly (Table 2). The value of parameter K increased more after gemfibrozil (-0.55 $\pm$ 0.18 to -0.32 $\pm$ 0.21; $p<0.001$), than after simvastatin (-0.55 $\pm$ 0.16 to -0.47 $\pm$ 0.22; $p=0.04$; gemfibrozil versus simvastatin $p<0.05$). In 5 of 45 patients treated with gemfibrozil, the dense subfraction profile was altered into an intermediate subfraction profile, with an equal amount of buoyant and dense LDL particles. The other patients retained a dense subfraction profile, expressed as a negative value for parameter K, despite lipid-lowering therapy.

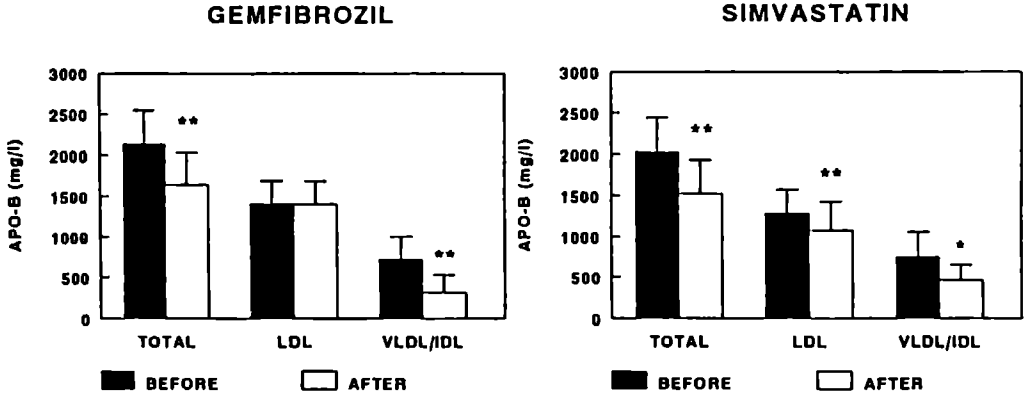

Figure 2: Effect of treatment with either Gemfibrozil (n=21) or Simvastatin (n=23) on apolipoprotein-B containing lipoproteins in total plasma, the VLDL+IDL fraction and the LDL fraction of patients with FCH; * $p=0.003$; ** $p<0.001$.

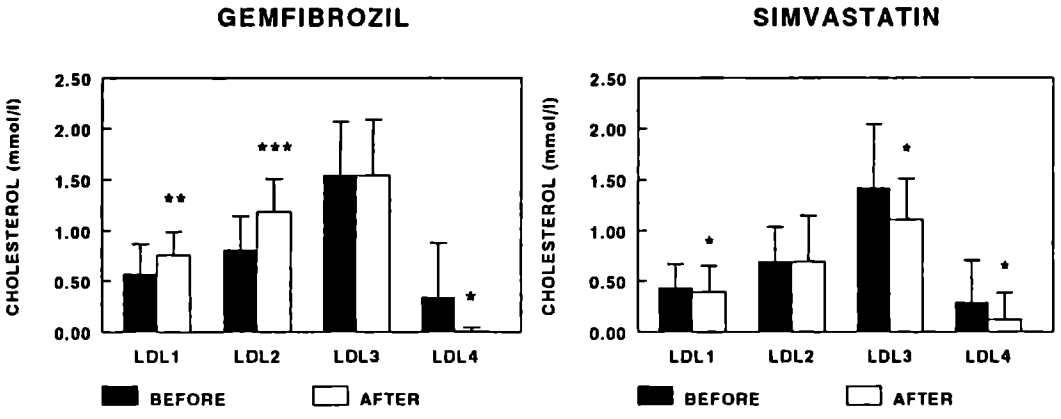

Figure 3: Effect of treatment with either Gemfibrozil (n=20) or Simvastatin (n=21) on the cholesterol content in mmol/l of four LDL subfractions (LDL1-LDL4) of patients with FCH; * $p<0.05$; ** $p=0.001$; *** $p<0.001$.

Table 2: Changes in the Ratios Cholesterol/Triglyceride, Cholesterol/Apo-B and Triglyceride/Apo-B of the LDL Particles After Treatment With Gemfibrozil (n=19) or Simvastatin (n=21) in Patients With Familial Combined Hyperlipidemia

	Medication	Before	After	Change (%)	p Value
Cholestrerol/	G	7.73 ± 2 27	10 26 ± 2.62	53.7 ± 67.0	0.001
triglyceride	S	6.14 ± 1 75	6.18 ± 1.87	1.6 ± 20.9′	
Cholesterol/apo-B	G	1.48 ± 0.17	1.54 ± 0.21	5.8 ± 22.0	
	S	1.34 ± 0.16	1.36 ± 0.16	0.5 ± 12.9	
Triglyceride/apo-B	G	0.47 ± 0.11	0 36 ± 0.08	-17.5 ± 19.3	<0.001
	S	0.53 ± 0.13	0 55 ± 0.17	5.0 ± 20.2†	

Values are presented as mean ± SD; G indicates gemfibrozil; S, simvastatin; Apo-B, apolipoprotein-B; ′$p<0.01$, †$p<0.001$; G versus S.

Fatty acid composition and vitamin E content of Low-density lipoprotein

The fatty acid composition of each isolated LDL was determined (Table 3). For technical reasons, only the results of 14 patients treated with gemfibrozil and 15 patients with simvastatin could be analyzed.

Table 3: Change in fatty acid composition and vitamin E content of total LDL after treatment with gemfibrozil or simvastatin in FCH patients

Fatty acid	Medication	Before	After	Change (%)	p
Palmitic acid	G	22.3 ± 1.9	21.7 ± 1.6	-2.8 ± 6.2	
(16:0)	S	23.4 ± 2.2	23.5 ± 2.4	+0.2 ± 5.7	
Stearic acid	G	6.6 ± 0.8	6.3 ± 0.7	-3.1 ± 5.9	0.04
(18:0)	S	6.6 ± 0.6	6.8 ± 0.7	+2.8 ± 6.3*	
Oleic acid	G	18.7 ± 1.9	17.8 ± 1.6	-4.8 ± 5.7	0.01
(18:1)	S	18.8 ± 1.8	19.0 ± 2.2	+0.9 ± 8.6*	
Linoleic acid	G	44.1 ± 3.7	45.8 ± 3.2	+4.1 ± 4.3	0.01
(18:2)	S	43.4 ± 3.6	41.6 ± 4.1	-4.0 ± 6.0‡	0.04
Arachidonic	G	8.2 ± 1.7	8.4 ± 1.6	+3.0 ± 14.5	
acid (20:4)	S	7.8 ± 1.4	9.1 ± 1.4	+20.1 ± 21.0†	0.01
Vitamin E[a]	G	4.06 ± 1.65	3.42 ± 1.66	-16.5 ± 18.2	0.004
	S	3.65 ± 1.76	3.82 ± 1.68	+14.0 ± 45.9*	
PUFA/Vitamin E[b]	G	846 ± 596	1102 ± 673	+42 ± 83	
	S	801 ± 538	727 ± 339	-6 ± 52	

Values of fatty acids are presented in percentage of total fatty acids as mean ± SD; G indicates gemfibrozil (n=14); S, simvastatin (n=15); [a]vitamin E in mg/g LDL protein; [b]ratio of poly unsaturated fatty acids/vitamin E in μmol/mg; *$p<0.05$, †$p<0.01$, ‡$p<0.001$, G versus S.

This reduction had no effect on lipid levels, apo-B levels, or the value of parameter K before and after therapy in this subset, if compared with the initial 45 individuals. In the gemfibrozil group the relative amount of stearic acid (18:0) and oleic acid (18:1) decreased, whereas that of linoleic acid (18:2) increased. In the simvastatin group the relative contribution of linoleic acid (18:2) decreased, with an increase of arachidonic acid (20:4). Vitamin E in LDL decreased significantly with gemfibrozil and was unaffected with simvastatin. The ratio polyunsaturated fatty acids/vitamin E tended to increase after gemfibrozil, whereas simvastatin did not affect this ratio.

Total LDL oxidizability

Because of technical reasons the results of only 17 patients treated with gemfibrozil versus 18 patients with simvastatin could be analyzed. This reduction had no effect on lipid and apo-B levels, or the value of parameter K before and after therapy in this subset, if compared with the initial 45 individuals. Although the lag time as preoxidative phase tended to increase, the differences were not significant in any of treatment groups. Oxidation rate decreased after simvastatin ($p=0.01$), in contrast to gemfibrozil. Total amounts of produced dienes per milligram of LDL protein were similar before and after treatment in both groups.

DISCUSSION

The underlying cause of the increased tendency toward cardiovascular diseases in patients with familial combined hyperlipidemia is probably related to increased levels of small dense LDL and other atherogenic lipoprotein remnant particles (8,14,21). A predominance of small dense LDL is observed either as a physiologic response to lipid abnormalities (22), or as a distinct characteristic of the disease with a possible genetic basis (17,23). These small dense subfractions are associated with atherosclerosis because of enhanced susceptibility to copper-induced oxidative modification (14). In the present study we therefore investigated the apo-B containing lipoprotein concentrations, LDL subfraction profiles and LDL oxidizability of 45 affected patients, and evaluated the effects of pharmacologic intervention on these parameters.

The observed reduction of total plasma cholesterol and plasma triglyceride concentrations with simvastatin (-22.2% and -15.9%, respectively) and gemfibrozil (12.9% and -48.1%, respectively) after 12 weeks of treatment are in accordance with previous reports (24,25). Isolation of VLDL and IDL particles together ($d < 1.019$ g/ml) instead of isolating IDL together with LDL ($d > 1.006$ g/ml), explains the relatively large contribution of VLDL+IDL cholesterol and the relatively small contribution of LDL cholesterol to total plasma cholesterol and also the large impact of the 2 therapies on this VLDL+IDL fraction. The increase of HDL cholesterol with simvastatin similar to gemfibrozil, despite a less pronounced reduction of triglyceride concentration after simvastatin, is larger than previously reported (25,26). The decrease and increase in LDL cholesterol with simvastatin and with gemfibrozil, respectively, are also in line with other reports (12,13,26). A depletion of triglycerides in the VLDL fraction by gemfibrozil, leading to small, more dense VLDL+IDL particles that are more likely to be converted into LDL particles, is suggested to be a cause of the observed increase in LDL cholesterol after gemfibrozil (26,27).

All patients had moderate to severe elevations of apo-B levels in accordance with their elevation of lipid levels. In normolipidemic subjects total apo-B related cholesterol concentration correlates highly with serum apo-B. In hypertriglyceridemic states this correlation is less pronounced because of possible underestimation of apo-B (28), although the correlation between total cholesterol minus HDL cholesterol and apo-B in these patients was still significant. Both therapies reduced total apo-B to a similar extent, but just like the reduction of VLDL+IDL cholesterol/triglyceride and LDL cholesterol, gemfibrozil reduced only VLDL+IDL apo-B containing particles and simvastatin reduced both VLDL+IDL and LDL apo-B containing particles.

Initially all patients had a dense LDL subfraction profile, both determined by cholesterol content in isolated LDL subfractions and described by a continuous variable, parameter K. This method of approach provides the opportunity to obtain more detailed information about small alterations in the LDL subfraction profile than the often used dichotomous classification in pattern A (light) and pattern B (heavy) (17). The increase of cholesterol in the buoyant LDL1 and LDL2 subfractions after gemfibrozil, could be

explained by the observed decrease of the ratio triglyceride/protein of the LDL particle, only after gemfibrozil, reflecting an overall reduction of triglyceride in the LDL particles. This triglyceride reduction, not observed after simvastatin, is associated with more buoyant LDL subfractions (14). It is remarkable that despite the substantial reduction of plasma triglyceride concentrations, none of the patients with a dense LDL subfraction profile had complete conversion to a buoyant LDL subfraction profile. Only 5 patients had conversion to an intermediate profile. These results are supported by a recent study by Hokanson et al (29) in which they proposed that in familial combined hyperlipidemia small dense LDL and hypertriglyceridemia appear as interrelated but separate characteristics and regulated as separate processes.

In general, the observed LDL fatty acid composition in this FCH population was similar to that found in normal subjects (18). After both therapies only small alterations in this composition were seen. The total amount of polyunsaturated fatty acids (linoleic acid and arachidonic acid), most susceptible for oxidative modification (18), did not change. On the contrary, vitamin E as the major antioxidant in LDL was reduced only by gemfibrozil. This might have implications for total LDL oxidizability.

Our data show only little effect on LDL oxidizability after treatment, less than suspected on the basis of a more buoyant LDL subfraction profile (14). However some explanations for this lack of change in LDL oxidizability are possible: We determined LDL oxidizability in total LDL, which is the addition of maximal 5 LDL subfractions, so small changes might be undetected. Despite treatment these patient still had a predominance of small dense LDL particles. The ratio cholesterol/protein of LDL particles correlating with LDL oxidizability (30) was unaffected after both therapies. Finally, the ratio polyunsaturated fatty acids/vitamin E increased only with gemfibrozil. This implies that the expected diminished susceptibility to copper-induced oxidation because of a more buoyant LDL subfraction profile (14) could be offset by a reduced protection of polyunsaturated fatty acids from oxidation by vitamin E.

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Chapter 4

Non-obese Patients with Familial Combined Hyperlipidemia are Insulin Resistant as Compared with their Non-affected Relatives

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ABSTRACT

Familial combined hyperlipidemia (FCH) is a heterogeneous lipid disorder, caused by overproduction of very low density lipoproteins and characterized by the occurrence of small dense low density protein (LDL) particles, all features that are also associated with insulin resistance. Therefore, insulin sensitivity was examined directly by means of the euglycemic hyperinsulinemic clamp technique in male non-obese, normotensive FCH patients and compared with that of their non-affected relatives, matched for age and body mass index (BMI). In addition, an oral 75 gram glucose tolerance test (OGTT) was performed and lipid values, including the LDL subfraction profile, were determined. During the clamp, forearm blood flow (FBF) was measured by venous occlusion plethysmography. All participants had a normal glucose response after the glucose load, whereas FCH patients showed hyperinsulinemia after OGTT and higher fasting C-peptide levels. During the clamp insulin concentrations increased equally in both groups. Mean whole body glucose uptake (M) (120-180 minutes) was lower in FCH patients compared with the non-affected relatives (6.89 ± 0.31 vs. 8.94 ± 0.76 mg/kg.min; $p=0.01$). Also the glucose uptake per unit insulin (I) was lower in FCH patients (M/I 7.46 ± 0.50 vs. 9.51 ± 0.53; $p=0.009$). Whole body glucose uptake significantly correlated with BMI, plasma cholesterol and triglyceride concentrations and the individual LDL density. The FBF correlated with insulin sensitivity and increased significantly in non-affected relatives (1.9 ± 0.12 to 2.5 ± 0.4 ml/min.dl; $p=0.025$), but not in patients. Thus, FCH patients characterized by a predominance of small dense LDL are insulin resistant as compared with their non-affected relatives. This insulin resistance may partly be explained by a decreased insulin-induced vasodilation in skeletal muscle.

INTRODUCTION

Familial combined hyperlipidemia (FCH) is a common heritable and heterogeneous lipid disorder, characterized by the presence of a multiple type hyperlipidemia with elevated plasma cholesterol and/or plasma triglyceride levels, and the frequent occurrence of premature cardiovascular disease in first-degree relatives (1-3). Originally, FCH was supposed to be caused by the variable expression of a single autosomal dominant gene primarily regulating plasma triglyceride levels and secondarily affecting cholesterol levels (1). However, several metabolic and biochemical defects that have recently been related to the trait suggest that the genetic basis of FCH is more heterogeneous. As a consequence, the spectrum of FCH also comprises other related phenotypes such as hyperapobetalipoproteinemia (4), the low density lipoprotein (LDL) subclass pattern B phenotype (5), familial dyslipidemic hypertension (6) and some features of the insulin resistance syndrome (7-9).

Insulin resistance, defined as a decreased ability of insulin to stimulate glucose uptake, is increasingly recognized as a common factor underlying various conditions, all of which predispose to coronary heart disease (10). Resistance to normal action of insulin is related to alterations in lipid metabolism such as an excessive postprandial release of non esterified fatty acids (NEFA), due to impaired suppression of hormone sensitive lipase activity (11). An increased supply of fatty acids to liver cells is associated with very low density lipoproteins (VLDL) overproduction (12,13), whereas an impaired activation of lipoprotein lipase adds to reduced clearance of triglyceride-rich lipoproteins (14). In addition, insulin resistance may coincide with a predominance of small dense LDL particles (6,15,16), although countered by others (17). Since these features (i.e., VLDL overproduction, impaired lipoprotein lipase activity and a predominance of small dense LDL), are also characteristics of FCH, the existence of insulin resistance may be an important factor modulating FCH phenotypes. The interpretation of data concerning the association between insulin sensitivity and lipid disturbances is, however, hampered by the fact that several features related to lipid disorders, as visceral obesity, age, gender, hypertension and existence of cardiovascular disease themselves also are associated with insulin resistance. Furthermore, hyperinsulinemia, as a consequence of insulin resistance, has been directly associated with cardiovascular disease (18).

Recent reports hypothesized that diminished glucose uptake may partly be explained by a decreased insulin-induced vasodilation in skeletal muscle (19,20). Furthermore, insulin's effect on vascular tone has been reported to be nitric oxide dependent (21). Interestingly, in dyslipidemic patients, endothelium-dependent vasodilation has been shown

to be diminished (22,23).

Several earlier reports have suggested the presence of insulin resistance in various forms of hyperlipidemia. However, these findings were either based on rather indirect measurements such as determination of plasma concentrations of glucose, insulin and NEFA (8), possible confounded by obesity of the study group (9), or obtained by steady-state plasma glucose (SSPG) and insulin (SSPI) concentration determinations after somatostatin administration (7). Somatostatin in itself may exhibit vascular effects. Therefore, in this study we have determined the sensitivity to the metabolic and hemodynamic effects of insulin directly by means of the euglycemic hyperinsulinemic clamp technique in combination with skeletal blood flow measurements. The studies were performed in a careful selected sample of well defined non-obese normotensive male FCH patients, which were compared with each of their matched non-affected relative.

SUBJECTS AND METHODS

Subjects

For this study, initially 12 unrelated non-obese normotensive male FCH patients, selected from an available sample of well defined Dutch FCH families, were compared with one of their non-affected male relatives. To anticipate environmental influences we carefully matched related individuals (i.e., affected and unaffected) for age, body mass index and waist to hip ratio. In addition, all subjects were apparently healthy and met the following inclusion criteria: a body mass index below 27 kg/m^2, blood pressure below 165/90 mmHg (measured in the supine position after 5 minutes rest), absence of diabetes mellitus, a medical history of vascular disease, and use of any medication, except lipid-lowering drugs, which were discontinued at least 1 month before participation. Two FCH patients and 2 relatives were habitual smokers.

The FCH diagnosis was based on the following criteria: (i) the presence of a multiple type hyperlipidemia in first-degree relatives, with at least two first-degree relatives with a different hyperlipidemia (i.e., hypertriglyceridemia, hypercholesterolemia or a combined hyperlipidemia). The assessment of hyperlipidemia was based on the presence of constant elevated cholesterol and/or triglyceride levels above the 90th percentile, in spite of dietary advice (24); (ii) a positive family history of premature cardiovascular disease before the age of 60 years.

Participating FCH patients had both total plasma cholesterol and triglyceride concentration above the 90th percentile for age and gender, following dietary advice and after withdrawal of lipid-lowering drugs for at least 4 weeks. In contrast, unaffected

relatives (9 were brothers, 2 were cousins) had both total plasma cholesterol and triglyceride concentration below the 75th percentile for age and gender. None of the subjects was homozygous for the apo E2 allele.

Protocol and Procedures

All participants were asked to visit the department twice after an overnight fast. During a first visit, a questionnaire was filled out in order to collect information on their medical status, and supine blood pressure, body mass index as well as the waist-hip ratio were measured. Further, blood was sampled for determination of baseline values for lipids, lipoproteins, HbA_{1C} and C-peptide and for determination of the individual distribution of LDL subfractions in the LDL subfraction profile. Moreover, a 75 gram oral glucose tolerance test (OGTT) was performed to exclude impaired glucose tolerance or overt diabetes mellitus. In these tests glucose and insulin concentrations were determined at baseline and 30, 60, 90 and 120 minutes after the glucose load. Only when participants showed 2-hour post-load glucose levels below 7.8 mmol/l, a hyperinsulinemic euglycemic clamp was performed, combined with hemodynamic measurement of blood pressure, heart rate and forearm blood flow, during a second visit, one to two weeks later. After OGTT, one FCH patient and his related control were excluded from further analyses due to impaired glucose tolerance of this FCH patient (2-hour glucose level of 10.2 mmol/l). The experimental protocol was approved by the ethical committee of our Hospital, and all participants gave their written informed consent.

Euglycemic Hyperinsulinemic Clamp

Experiments were performed with the participants in the supine position in a quiet, temperature-controlled room (24-25°C). Under local anaesthesia (0.3-0.4 ml lidocaine HCL 20 mg/ml), a 20-gauge catheter (Angiocath®, Deseret Medical Inc., Becton Dickinson and Comp., Sandy, Utah, USA) was inserted into the left brachial artery and connected with an arterial pressure monitoring line (Viggo Spectramed, No 992399A/14368, Bilthoven, The Netherlands) to a Hewlett Packard monitor (type 78353B, Hewlett Packard GmbH, Böblingen, Germany). Mean arterial blood pressure (MAP) was determined by electronically integrated area under the brachial arterial pulse-wave form. The arterial line was kept patent with saline infusion (with 2 U heparin/ml added). On the contralateral side an identical venous catheter was inserted into a large forearm vein for infusion of insulin and glucose. Insulin (Actrapid®, Novo-Nordisk, Denmark) was infused in a dose of 60 mU/m^2/minute for 180 minutes. Insulin was diluted in 50 ml 0.9% NaCl

with addition of 2 ml albumin, to a concentration of 1 U/mL. According to the clamp technique (25), plasma glucose concentration was kept at an euglycemic level by a variable infusion of glucose 20% solution, adjusted by arterial plasma glucose levels measured at 5 minutes intervals. All subjects were clamped at fasting glucose levels minus 0.3 mmol/L. The glucose infusion rate was calculated as the given amount of glucose in mL/kg body weight per minute, which equals the whole body glucose uptake (M). The glucose uptake per plasma unit of insulin (I) (insulin sensitivity index, M/I) was calculated as an additional measure of insulin sensitivity. In addition, the concentrations of non-esterified fatty acids (NEFA) were determined before and at the end.

Forearm Blood Flow Measurement

Forearm blood flow (FBF) was measured using mercury-in-silastic strain-gauge venous occlusion plethysmography on both arms as previously described (26). One minute before the start of the measurements (every 30 minutes), a wrist cuff was inflated to 100 mmHg above the systolic blood pressure, to be sure that the measurement only referred to the forearm skeletal muscle vascular bed (27). The collecting cuff around the upper arm was inflated to a pressure of 45 mmHg during 8-10 heart cycles using a rapid cuff inflator (Hokanson E20 rapid cuff inflator, Hokanson Inc., Issaquah, Wa, USA). The strain gauges were connected to a plethysmograph (Hokanson EC4 plethysmograph, Hokanson Inc., Issaquah, WA, USA). Changes in FBF during the clamp procedure were calculated with the means of the left and right FBF.

Analytical Methods

Glucose concentrations were measured in duplicate using the oxidation method (Beckman®, Glucose Analyser2, Beckman Instruments Inc., Fullerton, CA 92634, USA). Plasma insulin concentrations were determined using an "in house" assay (double antibody method) with an inter-assay variability of 6%. C-peptide was determined using a commercially double antibody kit (Diagnostic Products Cooperation, cat no KPED1 double antibody, CA 900455597, USA), with an inter-assay variability of 4.3%. Hemoglobin A_{1c} was determined with a HPLC technique (BIORAD Laboratories B.V. Veenendaal, The Netherlands), with a reference range of 4.8-6.2%. The concentrations of NEFA's were determined using a commercially available ACS-ACOD method (Waco Chemicals GmbH cat. no. 994-75409, Neuss, Germany). Total plasma cholesterol and triglyceride concentrations were determined by commercially available, enzymatic reagents (Boerhinger-Mannheim, FRG, cat. no. 237574 and Sera Pak, Tournai, Belgium cat. no.

6639, respectively). VLDL was isolated from whole plasma by sequential ultracentrifugation at density 1.019 g/ml for 16 hours at 36,000 rpm in a fixed angle rotor (TFT 45.6 rotor, Kontron, Zürich, Switserland) in a Beckman L7-55 ultracentrifuge (Beckman, Palo Alto, USA). High density lipoprotein (HDL) cholesterol was determined with the polyethylene glycol 6000 method (28). LDL cholesterol was calculated by subtraction of VLDL and HDL cholesterol from total plasma cholesterol. Total plasma apolipoprotein-B was determined by immuno-nephelometry (29). To achieve accurate results in relation to the Center for Disease Control Standardization Program, the obtained plasma apo-B results were recalculated on the basis of exchange of sera with Dr. S. Marcovina (Northwest Lipid Research Laboratory, Seattle, WA, USA). LDL subfractions were detected by single spin density gradient ultracentrifugation according to a previously described method (30). LDL subfractions were detected by single spin density gradient ultracentrifugation, according to a method described elsewhere (31). After ultracentrifugation up to five LDL subfractions, stained with Coomassie Brilliant Blue R, were visible as distinct bands in the middle of the tube. Accurate documentation of the different LDL subfraction patterns was obtained by scanning slides of the tubes in triplicate on a LKB 2202 ultrascan laser densitometer (Pharmacia LKB). The mean peak heights (h1-h5) of the LDL subfractions (LDL1-LDL5) on the three scans were used to calculate a continous variable K ($-1 < K < 1$), as described elsewhere (32). This continuous variable K appeared to reflect appropriately the individual LDL subfraction profiles (32,33). A negative K-value ($-1 < K < 0$) reflects a LDL subfraction profile more or less predominated by small dense subfractions. A profile with a predominance of buoyant LDL subfractions reveals a positive K value ($0 \leq K < 1$).

Statistical Analysis

Differences in baseline characteristics and baseline values for lipid, lipoproteins, glucose and HbA_{1c} were tested by the nonparametric two-sample Wilcoxon signed rank test. The results of the glucose tolerance test were tested by comparing areas under the curve of both the glucose excursion curve and the insulin response. To compare the whole body glucose uptake and the insulin sensitivity index of the affected with that of the unaffected subjects, controlling for possible influence of confounding variables, an analysis of variance (ANOVA) was performed with age, body mass index and waist to hip ratio as covariates. To investigate differences in the time action of insulin on hemodynamic parameters between the two groups, the time/group interaction was calculated by repeated measures ANOVA with insulin as the dependent factor. All

statistical analysis were performed using the SPSS/PC+ program (SPSS Inc, Chicago, Ill, USA). Results in Tables and Figures are presented as mean±SEM unless otherwise indicated. Differences with a test value less than 0 05 were considered to be significant.

RESULTS

Baseline Characteristics

Baseline characteristics, of both 11 FCH patients and their 11 non-affected relatives, are presented in Table 1 Age, BMI, waist to hip ratio and blood pressure were not different between both groups As a consequence of the selection procedure, all lipid and lipoprotein concentrations (except the LDL cholesterol concentration) were significantly higher in FCH patients compared with the unaffected relatives

Table 1: Baseline anthropometric measurements, blood pressure, lipid, lipoprotein and apolipoprotein-B levels of the FCH patients and their non-affected relatives

	Affected	Unaffected	p-value
Number	11	11	
Age (years)	41 9 ± 9 0	44 9 ± 11 5	ns
Body mass index (kg/m^2)	24 9 ± 1 2	24 3 ± 1 5	ns
Waist/hip ratio	0 99 ± 0 05	1 00 ± 0 04	ns
Systolic blood pressure (mmHg)	122 7 ± 11 7	132 7 ± 16 5	ns
Diastolic blood pressure (mmHg)	79 5 ± 6 9	81 8 ± 8 4	ns
Total cholesterol (mmol/L)	7 70 ± 1 01	5 59 ± 0 85	0 003
Total triglycerides (mmol/L)	3 73 ± 2 50	1 24 ± 0 43	0 003
VLDL cholesterol (mmol/L)	1 90 ± 1 40	0 50 ± 0 29	0 003
VLDL triglycerides (mmol/L)	2 80 ± 2 14	0 71 ± 0 41	0 003
LDL cholesterol (mmol/L)	4 90 ± 1 39	4 03 ± 0 88	ns
HDL cholesterol (mmol/L)	0 89 ± 0 17	1 20 ± 0 15	0 006
Apo-B (mg/dL)	178 9 ± 45 3	122 8 ± 26 8	0 003
K-value	-0 62 ± 0 13	-0 08 ± 0 24	0 004

All values are presented as means ± SD, VLDL, very low density lipoproteins, LDL, low density lipoproteins, HDL, high density lipoproteins, Apo-B, apolipoprotein-B, ns, not significant

Total plasma apo-B was also higher in FCH patients than in unaffected relatives. The K-value, as a description of the LDL subfraction profile, was more negative in FCH patients than in unaffected relatives, corresponding with the predominance of small dense LDL particles in FCH patients.

Responses to Oral 75 gram Glucose Load

Baseline values of fasting glucose, fasting insulin, HbA_{1c}, C-peptide, and the calculated insulin to glucose ratio are presented in Table 2. C-peptide concentrations were significantly higher in FCH patients compared with their unaffected relatives. Concentrations of glucose, insulin, HbA_{1c}, and insulin to glucose ratio all tended to be higher in FCH patients than in the unaffected, but these differences did not reach significant levels.

Table 2: Baseline concentrations of parameters of glucose metabolism in 11 FCH patients and 11 non-affected relatives.

	Affected	Unaffected	p-value
Glucose (mmol/L)	5.45 ± 0.12	5.22 ± 0.15	ns
Insulin (mU/L)	8.55 ± 0.95	7.00 ± 0.98	ns
HbA_{1c} (%)	5.45 ± 0.10	5.36 ± 0.08	ns
C-peptide (nmol/L)	0.65 ± 0.03	0.47 ± 0.04	0.02
Insulin to glucose ratio	1.57 ± 0.18	1.35 ± 0.18	ns
NEFA	0.86 ± 0.16	0.75 ± 0.05	ns

All values, except the insulin to glucose ratio, are presented as means ± SEM; NEFA, non esterified fatty acids; ns, not significant.

Glucose excursions after oral glucose load were comparable in both groups (Figure 1), although a significant higher glucose concentration after 2 hours was observed in FCH patients as compared with the controls (5.73±0.52 mmol/L vs. 4.36±0.29 mmol/L, p=0.019). Plasma insulin responses during 2 hours after the oral glucose load, also presented in Figure 1, were significantly higher in patients than in controls. None of the participants had plasma glucose above 7.8 mmol/L, 2 hours after an oral 75 gram glucose load.

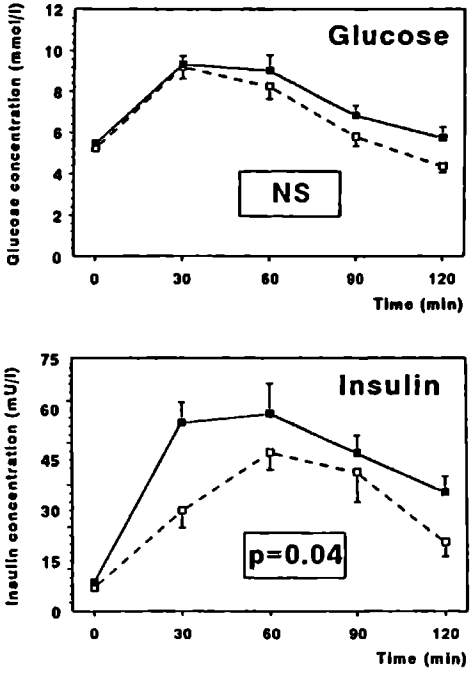

Figure 1: Plasma glucose (top) and insulin (bottom) concentrations in response to the oral 75 gram glucose tolerance test in 11 FCH patients (■) and 11 unaffected relatives (□). For glucose response, calculated areas under the curve were not significantly different, but mean glucose concentration at 120 minutes was significantly higher in patients than in controls. For insulin response, calculated areas under the curve were significantly higher in FCH.

Metabolic Response to Clamp Procedure

Plasma glucose values during the last 60 minutes of the clamp procedure were stable in patients and unaffected relatives (coefficient of variation 3.4±0.3% and 4.1±0.3%, respectively). Plasma insulin concentrations increased in all subjects and were similar after 3 hours of insulin infusion in both groups (94.6±4.3 mU/L in FCH patients and 93.8±4.8 mU/L in unaffected relatives). Whole body glucose uptake (M), calculated from the glucose infusion rate of both groups during the clamp procedure, is presented in Figure 2, and was significantly different over the last 60 minutes between FCH patients and unaffected relatives (6.89±0.31 vs. 8.94±0.76 mg/kg.min, respectively; p=0.01 ANOVA). The mean M-value of the control relatives was very similar to previous obtained values of healthy volunteers (8.8±1.3 mg/kg.min) (34). The glucose uptake during the last 60 minutes of the clamp procedure per plasma unit of insulin (insulin

sensitivity index) also differed between both groups (M/I 7.46±0.50 vs. 9.51±0.53, respectively; p=0.009 ANOVA).

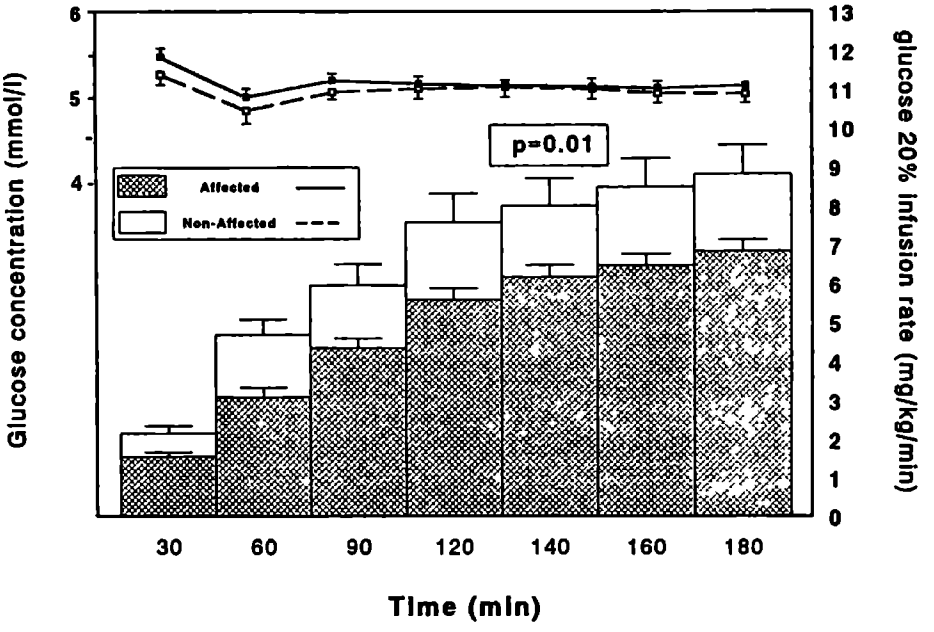

Figure 2: Results of the euglycemic hyperinsulinemic clamp procedure during 3 hours in 11 FCH patients and 11 unaffected relatives Left Y-axis Mean glucose concentrations of FCH patients (■) and unaffected controls (□) presented in the upper lines Right Y-axis Significantly different mean glucose infusion rate (mg/kg min) as measure for glucose uptake during the clamp procedure of affected (closed bars) and unaffected (open bars)

When the results of the related couples were compared it was found that in 9 FCH patients whole body glucose uptake was lower, in 1 FCH patient it was equal and in 1 FCH patient it was higher than in their respective normolipidemic relatives (Figure 3). To avoid the possible confounding effect of smoking, the rates of uptake were also compared in non-smokers. In non-smokers the rates of whole body glucose uptake were also significantly lower in FCH patients (n=9) than in unaffected relatives (n=9) (6.90±0.66 vs. 9.00±0.93 mg/kg min, respectively; p=0 019 ANOVA). Although fasting plasma NEFA concentrations were higher in the FCH patients than in controls, this difference appeared not to be significant (Table 2). Both NEFA concentrations were suppressed in a

similar manner during the clamp procedure (from 0.86±0.16 to 0.12±0.04 mmol/L in FCH patients, p=0.003 and from 0.75±0.12 to 0.10±0.05 mmol/L in unaffected relatives, p=0.003), without differences between both groups.

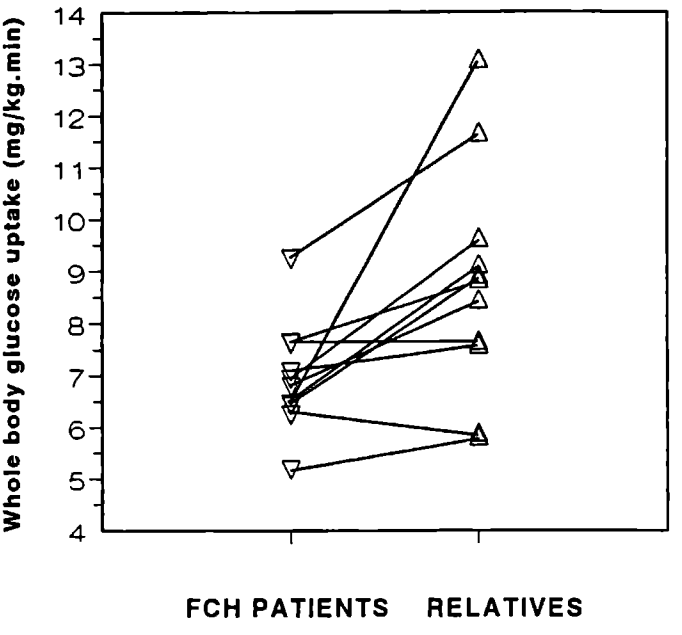

Figure 3: Comparison of whole body glucose uptake during clamp procedure of related couples (i.e., FCH patients and relatives) connected by lines.

Effects on Forearm Blood Flow

At baseline, the mean values of hemodynamic parameters (FBF and MAP) of both groups were similar. Only unaffected relatives exhibited a significant increase of 31.8±14.3% in forearm blood flow (FBF from 1.85±0.16 to 2.45±0.35 ml/dl.min, ANOVA p=0.025), whereas FCH patients showed no significant change in forearm blood flow (1.87±0.15 to 2.06±0.24). MAP did not change in either group (FCH patients: 92.7±2.3 to 93.0±3.6 and relatives: 95.3±3.0 to 98.4±2.7 mmHg). The time course of the changes in FBF showed the major and statistically significant changes during the last hour of the clamp reflecting a phenomenon with slow-onset.

Relationship between LDL subfraction distribution and whole body glucose uptake

The individual LDL subfraction profile, described as parameter K, correlated significantly with the glucose uptake during the clamp procedure (Pearson's r=0.51, p=0.008) (Figure 4).

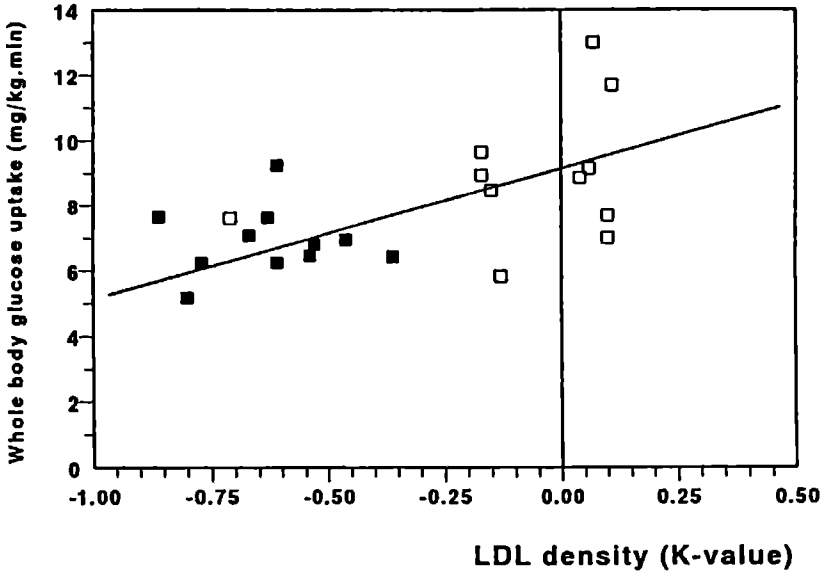

Figure 4: Correlation between glucose uptake during the clamp procedure and LDL density as determined by density gradient ultracentrifugation and described by parameter K. FCH patients are depicted by a closed box (■) and unaffected relatives by an open box (□) (r=0.51, p=0.008).

When the groups were compared separately, FCH patients were more insulin resistant and exhibited a predominance of small dense LDL subfractions. The unaffected relatives, with the exception of one individual, were characterized by the presence of more buoyant LDL particles.

DISCUSSION

In this study, we demonstrated by means of the euglycemic clamp technique directly a reduced insulin sensitivity in well-defined male FCH patients as compared with one of their normolipidemic relatives. Since it has been established that insulin resistance is also associated with obesity, age and gender alone (35,36), it is important to emphasize that

these observations were not confounded by differences in gender or age, and were registered in the absence of obesity, hypertension and impaired glucose tolerance in the participants. Furthermore, we selected the affected and the unaffected participants in genetically related pairs, presuming that by this study design differences in environmental or genetic influences were reduced. Apart from the metabolic resistance to insulin (reduced glucose uptake), FCH patients also exhibited resistance to the vasodilator effects of insulin. Finally, diminished whole body glucose uptake correlated with the presence of small dense LDL subfractions.

Because of the similarity in presenting lipid disturbances and the association with increased cardiovascular risk, diminished insulin sensitivity may be related to FCH. This observation is further supported by the finding that insulin resistance affects both hepatic VLDL production and lipoprotein lipase action features also met in FCH. Previous studies indicate that insulin resistance is associated with elevated concentrations of VLDL triglyceride (7,37,38). Recent studies, however, showed no improvement in insulin sensitivity after substantial reduction of plasma triglyceride levels with fibrate treatment in both non-diabetic men with type IIb hyperlipidemia and in type II diabetic patients with hypertriglyceridemia (39,40). This may indicate that in some patients insulin resistance is of an inherited nature, not depending on plasma triglyceride concentrations, but probably preceding VLDL triglyceride overproduction.

A proposed mechanism responsible for VLDL overproduction is the impaired postprandial suppression of NEFA release in adipose tissue due to resistance to the normal suppressive effect of insulin on hormone sensitive lipase (11,41). An elevated concentration of NEFA is associated with increased hepatic VLDL triglyceride secretion, possibly due to reduced intracellular apo-B degradation (12). Furthermore, when normal insulin mediated activation of lipoprotein lipase in adipose tissue is diminished, an impaired postprandial clearance of triglyceride-rich lipoproteins may occur (14). Several reports mentioned an impaired lipoprotein lipase activity as one of the factors contributing to FCH (42,43).

A postprandial status with prolonged circulating triglyceride-rich lipoproteins is a provocating situation for the formation of small dense LDL particles (44). In the Kaiser Permanente Women Twin study and other studies small dense LDL was found to be an integral feature of the insulin resistance syndrome (15,16). Others, however, showed a significant correlation between LDL particle size and triglyceride levels, but not to insulin resistance in mildly hypertriglyceridemic subjects (17). Here, the authors considered small dense LDL a feature of the insulin resistance syndrome more as a consequence of

abnormalities in VLDL metabolism. Our observed correlation between glucose uptake as a measure of insulin sensitivity and LDL density is in accordance to these reports, but cannot discriminate between metabolic influences, such as the correlation with hypertriglyceridemia, causing a dense LDL subfraction profile, or primarily genetic effects related to diminished insulin sensitivity and small dense LDL in FCH. The recently reported major gene effect on LDL subfraction size in FCH (5,33), may possibly be found in mutations causing insulin resistance underlying hypertriglyceridemia and the formation of small dense LDL. On the other hand, reported linkage of small dense LDL to a locus near the LDL receptor and the insulin receptor gene on chromosome 19p (45) may indicate that the allele responsible for expression of small dense LDL also predisposes to insulin resistance.

Disorders related to insulin resistance seems to exhibit their major defect on the level of glucose uptake in skeletal muscle, rather than at the level of the liver or the adipose tissue (46-48). Furthermore, several groups including ourselves have demonstrated the vasodilator effect of systemic hyperinsulinemia (19,34,49), which was reduced in non-insulin-dependent diabetes (NIDDM) (50). It is hypothesized that decreased insulin sensitivity in humans may not only be due to lower insulin-mediated glucose extraction in insulin-sensitive tissues, but also to a lower blood flow to these tissues, due to a decreased ability of insulin to stimulate skeletal muscle blood flow (20). In accordance with other studies in which comparable systemic insulin concentrations were reached, we observed a 30% increase in FBF in non-affected, but not in FCH patients. Although part of the present reported insulin resistance may be related to this diminished insulin-induced vasodilation, this could in fact only account for the last 60 to 80 minutes of the clamp, since differences in FBF between the groups became statistically significant in this period.

The insulin-induced vasodilation seems to be endothelium-dependent (21). Noteworthy, reduced insulin-induced vasodilation may be due to direct endothelium disturbing action of elevated lipid levels (23). Therefore, in FCH patients the elevated lipoprotein concentration may directly influence the endothelium function, resulting in decreased nitric oxid release during hyperinsulinemia. Because insulin resistance itself may underlie VLDL overproduction, the reduced hemodynamic effects of insulin in hyperlipidemia can amplify metabolic disturbance as found in FCH patients.

Recently, the Insulin Resistance Artherosclerosis Study demonstrated an inverse association between insulin sensitivity and atherosclerosis, as assessed by measures of the carotid artery wall (51). This association was eliminated, although not completely, by correction for traditional cardiovascular risk factors. The authors suggested that insulin

resistance is indeed associated with several established cardiovascular risk factors (51), however, the opposite conclusion of atherosclerosis underlying insulin resistance was not drawn. Therefore, insulin resistance in our FCH patients, exposed to elevated lipid levels for a relatively short period of time (mean age less than 42 year) and without signs of atherosclerosis, represents more likely a metabolic defect than being the consequence of atherosclerosis.

In conclusion, this study suggests that FCH patients do exhibit a diminished insulin sensitivity. This insulin resistance may underlie the observed hyperlipidemia, characterized by elevated concentrations of VLDL cholesterol and triglycerides and a predominance of small dense LDL in the affected subjects, and may be partly explained by a reduced capacity of insulin to induce vasodilation in skeletal muscle of the patients. Therefore, interactions between insulin-action and lipid metabolism in FCH warrant further investigation.

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Chapter 5

The Lipoprotein Lipase (Asn291→Ser) Mutation is Associated With Elevated Lipid Levels in Families With Familial Combined Hyperlipidemia

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ABSTRACT

Familial combined hyperlipidaemia (FCH) is one of the major genetic causes of coronary heart disease and is characterised by elevated levels of plasma cholesterol and/or triglycerides in individuals within a single family. Decreased lipoprotein lipase (LpL) activity has been found in some cases of FCH. A recent study revealed a common mutation in the LpL gene, LpL(Asn291→Ser), with a frequency of 9.3% in Dutch FCH patients (Reymer et al., Circulation, 90 (1994) I-998). This mutation was found in 3 out of 17 FCH families. Extensive family studies were subsequently performed to determine the effect of this mutation on the phenotypic expression of FCH. Using a pedigree-based maximum likelihood estimate, we demonstrated that the LpL(Asn291→Ser) mutation significantly affects the levels of plasma and very low density lipoprotein (VLDL) triglycerides (2.03 ± 0.21 versus 1.14 ± 0.13 and 1.21 ± 0.16 versus 0.62 ± 0.09 mmol/l, carriers and non-carriers respectively) and VLDL- and high density lipoprotein cholesterol (0.83 ± 0.10 versus 0.38 ± 0.06 and 1.02 ± 0.08 versus 1.29 ± 0.05 mmol/l, carriers and non-carriers, respectively), but not those of plasma and low density lipoprotein (LDL) cholesterol. These findings indicate that the LpL(Asn291→Ser) mutation is associated with elevated lipid levels, indicating it may be one of the genetic factors predisposing to FCH in the families studied.

INTRODUCTION

Familial combined hyperlipidaemia (FCH) is commonly found among survivors of premature myocardial infarction. Goldstein et al. (1) were the first to show that the FCH syndrome was distinct from familial hypercholesterolaemia and familial hypertriglyceridaemia. FCH is one of the major genetic causes of coronary heart disease with an estimated frequency of 0.3-2% in the general population (1). FCH probands show elevated plasma levels of cholesterol, triglyceride, or both. The FCH phenotype may vary from time to time in a given patient or among affected relatives within a single family (2). In addition, characteristics such as increased very low density lipoprotein (VLDL) production, predominance of small dense low density lipoprotein (LDL), hypertension and insulin resistance have been described to be associated with FCH (3-5). Considering elevated levels of either VLDL, LDL or both as affected phenotype in family studies, FCH was initially suggested to be an autosomal dominant disorder (1,6). Recently, using complex segregation analysis, Cullen et al. (7) found evidence for a major gene acting on triglycerides in families with FCH.

Several genes, including the apolipoprotein B (APOB) gene, the APOAI-CIII-AIV gene cluster, the LDL receptor and the lipoprotein lipase (LpL) genes (8-13) have been suggested to be associated with the appearance of FCH. However, despite extensive studies in FCH families, a major genetic defect underlying this heterogeneous and possibly polygenetic disorder has not been reported.

Babirak et al. (12) showed in a study among relatives from homozygous LpL-deficient probands that the heterozygous state for LpL deficiency, determined by measurement of posthepharin LpL activity and mass, often segregates with hyperlipidaemia and decreased levels of high density lipoprotein (HDL) cholesterol. In addition, decreased LpL activity has been found in one third of the cases with FCH (13). These results suggest that heterozygosity for LpL mutations may be one of the factors influencing the lipid phenotype of FCH. Recently, the LpL(Asn291→Ser) mutation was identified in 9.3% of Dutch patients with FCH (14). The identification of this mutation in three unrelated FCH probands enabled us to study the inheritance of this mutation within FCH families and estimate its effect on the lipid levels. Statistical analysis showed that the LpL(Asn291→Ser) mutation significantly affects lipid parameters, implying that this LpL mutation may be one of the genetic factors contributing to the FCH phenotype in these families.

SUBJECTS, MATERIAL and METHODS

Subjects

FCH probands were selected from patients, attending the lipid clinics in Nijmegen and Amsterdam for analysis of a lipoprotein disorder, when they fulfilled the following criteria: (i) elevated levels of both total cholesterol and triglycerides (at first measurement), (ii) a personal or family history of premature cardiovascular disease, and (iii) at least one first degree relative with elevated total cholesterol and/or triglycerides levels. None of the FCH probands had specific clinical signs, like tendon xanthomata, and none were homozygous for the APOE*2 allele. For all probands, a secondary cause of hyperlipidaemia was excluded by standard laboratory tests. Using these criteria 17 probands were diagnosed to have FCH. The study protocol was approved by the ethical committee of the universities of Amsterdam and Nijmegen.

Lipid and lipoprotein analysis

Ethylenediamine tetraacetic acid (EDTA) blood samples were obtained from the three probands and family members after an overnight fasting. No lipid lowering drugs were administered to the subjects for 6 weeks at the onset of the study, except for individual II-5 of family A. This individual was still on medication when the blood samples were collected. Plasma was separated from cells by centrifugation at 500 *g* for 10 min at room temperature, and was used for lipid and lipoprotein analysis.

VLDL ($d < 1.006$ g/ml) was isolated by ultracentrifugation for 16 hours at 36,000 rpm in an fixed-angle TFT 45.6 rotor (Kontron, Zurich) (15). Plasma and lipoprotein cholesterol and triglyceride concentrations were determined by enzymatic, commercially available reagents (No. 237574; Boehringer-Mannheim, FRG; Sera-pak, No. 6639; Tournai, Belgium). HDL-cholesterol was determined in whole plasma using the polyethylene glycol 6000 method (16). LDL cholesterol was subsequently calculated using the formula of Friedewald et al. (17).

Detection of the LpL(Asn291→Ser) mutation

Genomic DNA was isolated from leucocytes according to Miller et al. (18). Identification of the LpL(Asn291→Ser) allele carriers in probands and their families was performed by polymerase chain reaction (PCR) using a mutagenic amplification primer approach. Primer LpL291L: 5'-*ATA ATA TAA AAT ATA AAT A*CT GCT TCT TTT GGC TCT GAC- TGTA-3' was designed with a nucleotide mismatch (underlined) as compared to the wild type LpL sequence (19). In the case of the mutant allele an *RsaI* restriction site

is introduced due to the germline missense mutation and the nucleotide mismatch in the primer. The primer was elongated with a TA-rich stretch (italics) at the 5' end to facilitate the subsequent electrophoretic screening (see below). PCR was performed using primer LpL291R 5'-GCC GAG ATA CAA TCT TGG *TA*-3' and primer LpL291L. The reaction mixture included 15 pmol of each primer, 0.5 μg genomic DNA, 0.2 mM of each dNTP, 10 mM Tris-HCl; pH 9.0, 1.5 Mm $MgCl_2$, 50 mM KCL, 0.01% (w/v) gelatin, 0.1% Triton X-100, 0.1 unit *Taq* polymerase (Super Taq, HT biotechnology Ltd, UK), and 10% dimethylsulphoxide (v/v) in a total volume of 50 μl. Amplification was performed for 32 cycli of 30 s at 94 °C, 30 s at 53 °C and 1 min at 72 °C, with an initial denaturation period of 3 min. Some 20 μl of PCR products were digested with the restriction enzyme *RsaI* according to recommendations of the supplier (Pharmacia). Thereafter, fragments were separated on a 4% MP agarose gel (Boehringer Mannheim, FRG) and stained with ethidium bromide. Digestion of PCR product will reveal two fragments for the mutant allele of approximately 240 and 40 bp, and one fragment of 280 bp for the normal allele.

Statistics

Studying three FCH families implies, in a strict sense, that there were only three independent observations which can be used for statistical analysis. To test for the statistical significance of the effect of the mutant LpL(Asn291→Ser) allele on the lipoprotein traits in these three families, we used a pedigree-based maximum likelihood method developed by Lange et al. (20). Using standard statistical analyses would give similar results concerning the univariate statistical calculations but inappropriate standard errors due to fact that individuals are related.

For a given pedigree of n individuals a vector of observations (x) is defined and a vector of expected values [E(x)], that can depend on measured variables such as gender or measured genotype. The covariance between the residual part of the observations, i.e., the part that is not accounted for by the measured genotype or other variables, depends on the relationship between the pedigree members and on the genetic model assumed for the observations. Throughout, we have modeled the variances not accounted for by the measured genotype as consisting of additive genetic and random environmental variance, recognizing that the genetic part may also reflect environmental influences shared by family members. However, our main interest is to test for the influence of the measured genotype. For a given E(x) and expected covariance matrix Σ, the log likelihood of obtaining the observation vector x is:

$L = -½\ln |\Sigma| - ½[x - E(x)]'\Sigma^{-1}[x - E(x)] + \text{constant}$, where ' denotes matrix transpose.

The joint log-likelihood of obtaining all pedigrees is the sum of the log-likelihood of the separate pedigrees. Estimation involves selection of parameter values under a specific model that maximizes the joint likelihood of all pedigrees. The likelihood obtained for different models can be compared with chi-squared difference tests where $X^2 = 2(L_1 - L_0)$ and L_1 and L_0 denote the log-likelihood for the general (H_1) and the constrained (H_0) hypothesis. The degrees of freedom (df) for this test are equal to the number of independent parameters between H_1 and H_0 (21). The Fisher package (19) was used for genetic modelling. Ascertainment correction was carried out by conditioning on the probands. Extensive description of model definition: A: most general model allowing for: (i) age regression, (ii) gender-difference, and (iii) difference between carriers and non-carriers; B: Equal to model A but no age differences; C: Equal to model A but no gender difference, except for the trait 'HDL-Chol' where model C is equal to model B but no gender difference; D: Equal to model C but no difference between carriers and non-carriers, except for the trait 'VLDL-Tg' where model D is equal to model A but no difference between carriers and non-carriers and the trait 'HDL-chol' where model D is equal to model B but no difference between carriers and non-carriers. Testing procedure: (1), Model B is tested against model A. The data from the probands were omitted from statistical calculations in order to avoid possible ascertainment bias.

RESULTS

Probands and families

As a part of an ongoing study aiming at the identification of genetic risk factors underlying FCH we screened 17 FCH probands for the presence of a common LpL mutation, LpL(Asn291→Ser). Screening revealed three, apparently unrelated, carriers. The lipid parameters of these three probands, at their first visit to a lipid clinic, are shown in Table 1. Family members with total cholesterol and/or triglyceride levels above the 90th percentile using the age- and sex-related percentile levels of the PROCAM study are indicated in Figure 1.

Family studies were performed for all three probands, including 67 relatives. During screening of these 3 families, 19 carriers for the LpL(Asn291→Ser) mutation were found, including an individual related by marriage. One of the offsprings of this marriage proved to be homozygous for the mutation (Figure 1).

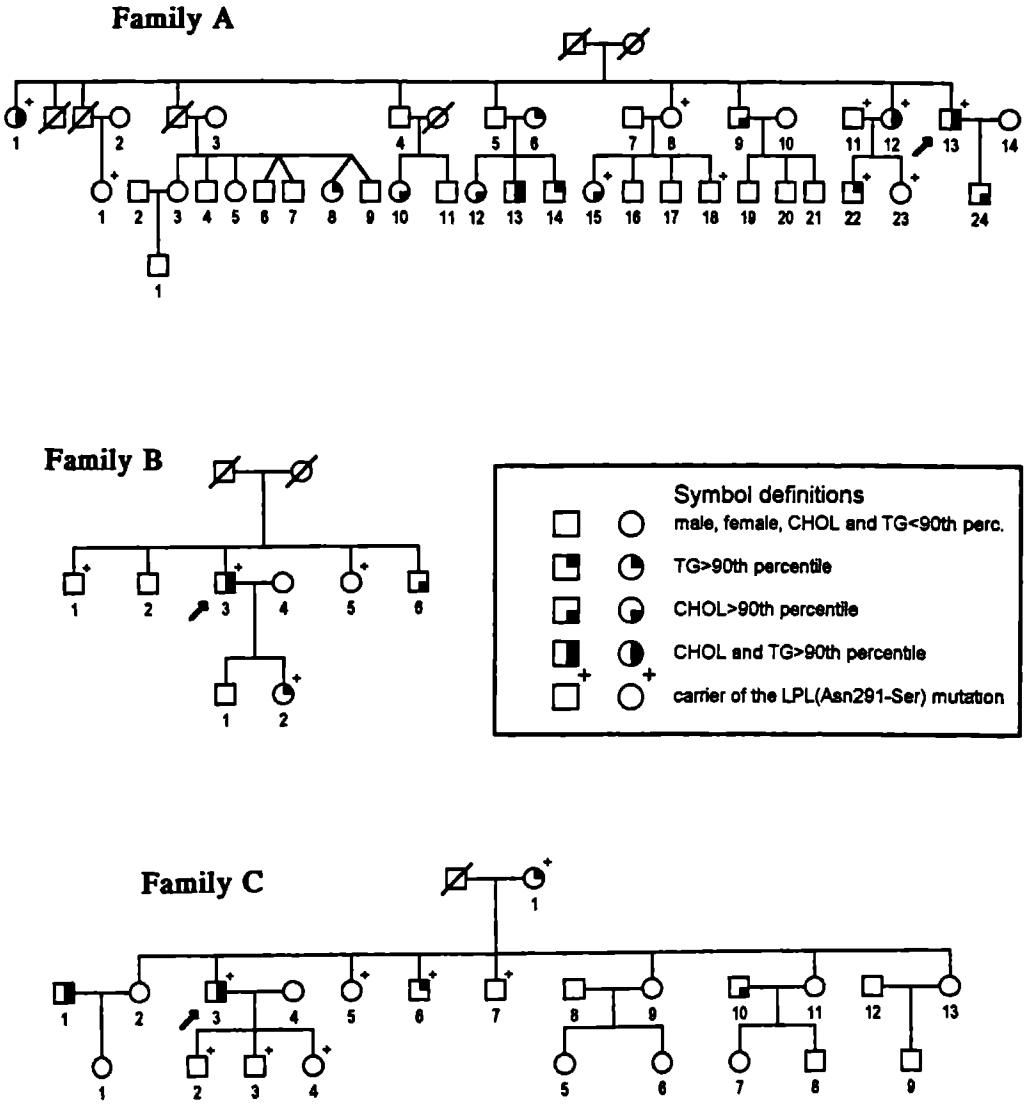

Figure 1: Pedigrees of the families of the three probands. Probands are indicated with an arrow. The symbol definitions are indicated in the figure. The homozygous carrier of the LpL(Asn291→Ser) mutation is relative number 22 of family A. Individual II-5 from family A was using lipid-lowering drug.

This homozygous carrier of the LpL(Asn291→Ser) mutation appeared to have hypertriglyceridaemia (total cholesterol: 4.8 mmol/l and total triglycerides: 3.1 mmol/l) at the age of 22 despite very lean body constitution (BMI 21 kg/m^2). Although total cholesterol levels are not elevated, the VLDL cholesterol is rather high, 1.44 mmol/l while HDL levels are decreased, 0.66 mmol/l. Screening 114 random individuals of a general Dutch population revealed no carriers suggesting that this mutation occurs with a low frequency in the general population.

Lipid and lipoprotein levels in carriers versus non-carriers

Plasma samples of the three probands and 67 additional family members were studied for lipid and lipoprotein parameters. The clinical details of allele carrying (n=19) family members, including the homozygous carrier, and non-carrying (n=48) family members are presented in Table 2.

Table 1: Clinical characteristics of three LpL(Asn291→Ser) probands.

	A:II-13	B:II-3	C:II-3
Age (yrs)	45	56	52
Gender	M	M	M
BMI (kg/m^2)	22.5	26.9	28,5
Plasma cholesterol (mmol/l)	7.6	8.40	9.50
Plasma triglycerides (mmol/l)	4.22	3.71	6,49
VLDL-cholesterol (mmol/l)	ND	1.98	ND
LDL-cholesterol (mmol/l)	ND	5.61	ND
HDL-cholesterol (mmol/l)	0.87	0.97	0.63

Plasma samples were collected after an overnight fasting.

The statistical analyses of these data are presented in Table 3. Compared to non-carriers, the LpL(Asn291→Ser) allele carriers exhibited markedly increased levels of plasma and VLDL-triglycerides (2.03±0.21 versus 1.14±0.13 and 1.21±0.16 versus 0.62±0.09 mmol/l, respectively) and VLDL-cholesterol (0.83±0.10 versus 0.38±0.06) (Table 2). HDL-cholesterol was slightly decreased in carriers (1.02±0.08 versus 1.29±0.05 mmol/l). As indicated in the Materials and Methods section, we considered allele carriers and non-carriers of three different families, implying that, in a strict sense,

there were only three independent observations.

Table 2 Descriptive statistics (means ± standard error) of the LpL(Asn291→Ser) allele carriers and their non-carrier relatives.

	LpL(Asn291→Ser) allele carrier relatives (n=19)	Non-carrier relatives (n=48)
BMI(kg/m^2)	24.91±0.83	23.76±0.52
Plasma TG#	2.03±0.21	1.14±0.13
VLDL-TG	1.21±0.16	0.62±0.09
Plasma Chol	5.85±0.29	5.34±0.18
VLDL-Chol	0.83±0.10	0.38±0.06
LDL-Chol	3.98±0.26	3.57±0.16
HDL-Chol	1.02±0.08	1.29±0.05

Means and asymptotic standard errors were estimated from the most general model using the maximum likelihood estimate procedures implemented in the Fisher program. Probands were excluded for these quantitative analyses. # all levels are expressed in mmol/l.

Therefore, to test for the effect of the mutant LpL(Asn291→Ser) allele in these families, we used a pedigree-based maximum likelihood method (20). The principle of this statistical method is briefly described in the Materials and Methods section. The legend of Table 3 indicates the four different models that were considered for the quantitative variables. Model A is the most general model, estimating the effects of age, gender and carrier status. Models B, C and D subsequently leave out each of these effects and are tested for a significant decrease in likelihood. In this table we present, when significant, the percentage of the total variance that can be explained by age, gender or carrier status, respectively. BMI did not differ between the two groups. Therefore, it cannot explain the differences found between carriers and non-carriers and is not used for one of the models. Table 3 shows that age influences BMI and all lipid parameters measured except HDL-cholesterol. In addition, gender influences levels of VLDL-triglycerides and HDL-cholesterol. No significant effects of additional genetic or environmental variances could be detected on the residual variance (results not shown).

Independently from the effects of age and gender, it is apparent that carrier status of the LpL(Asn291→Ser) mutation significantly affects the levels of plasma and VLDL-

triglycerides, VLDL- and HDL-cholesterol but not of plasma and LDL-cholesterol. In general, carrier status appears to explain approximately 14% of the total variance of these traits.

Table 3 Log-likelihood for four different models testing the effects of age, gender, and carrier status for the quantitative traits and BMI in the LpL(Asn291→Ser) pedigrees.

					Percent variance explained by		
	A	B	C	D	Age	Gender	Carrier status
BMI	-104.19	-119.51§	-104.21	-104.32	35.9	-	-
Plasma TG	-21.41	-25.92‡	-22.44	-26.09‡	9.9	-	12.9
VLDL-TG	-1.83	-5.17*	-4.04*	-5.43*	7.9	5.6	13.9
Plasma chol	-33.39	-47.16§	-34.57	-34.73	29.7	-	-
VLDL-chol	26.89	21.67§	25.99	22.24‡	11.5	-	13.5
LDL-chol	-27.25	-38.37§	-27.49	-27.60	24.6	-	-
HDL-chol	46.58	46.55	35.81§	40.90§	-	23.6	16.2

Log-likelihoods for four models. Model definition: A: most general model allowing for: (i) age regression, (ii) gender-difference, and (iii) difference between carriers and non-carriers; B: Equal to model A but no age differences; C: Equal to model A but no gender difference; D: Equal to model C but no difference between carriers and non-carriers. Testing procedure: (1), Model B is tested against model A. When twice the difference in log-likelihoods of these models is higher than the X^2 corresponding to df=1 this indicates a significant age difference (as indicated by * for $P < 0.05$ ($X^2>3.84$), ‡ for $P < 0.01$ ($X^2>7.88$) or § for $P < 0.001$ ($X^2>10.83$) (2) Model C (with df=1) is tested against model B as described above. When model B was significantly different from model A, then model C was tested against model A. (3) Model D (with df=1) is tested against model C or, in case of a significant effect of gender in model C, model B, or in case of a significant effect of age in model B, model A.

DISCUSSION

FCH is a frequently occuring lipid disorder in which multiple lipoprotein phenotypes occur within one family (1,6,7). Although, no major underlying defect has been found sofar, some studies suggested a link between heterozygosity for LpL deficiency and FCH (12,13). Therefore, we investigated the LpL gene as a candidate gene in Dutch FCH families.

We have screened FCH families for a common mutation, LpL(Asn291→Ser) which

occurs with a frequency of 9.3% among Dutch FCH patients (14). Moreover, in vitro site-directed mutagenesis revealed that the LpL(Asn291→Ser) mutation affects the catalytic function of LpL by 50% reduction of LpL activity and mass (22), making it a feasible candidate gene for FCH.

Three probands from our study carried the LpL(Asn291→Ser) mutation. This allowed us to determine the effect of this mutation on lipid and lipoprotein parameters within FCH families. We have used a pedigree-based maximum likelihood method described by Lange et al. (20) allowing to study the influence of measured alleles on quantitative traits under different models since the subjects included are not unrelated and derive from only three families. Since age and gender effects have an considerable influence on interindividual variability (23), we have estimated the effects of age and gender on the quantitative lipid traits and BMI for carriers and non-carriers. In addition to the influences of age and gender, the LpL(Asn291→Ser) allele appeared to contribute considerably (14%) to the total variance in VLDL and HDL levels. Recently, a segregation analysis of 55 FCH families, predicted a model for FCH in which a major gene, predominantly acting on triglycerides, would explain 20% of the phenotypic variance in triglyceride levels (7). The influence found for the LpL(Asn291→Ser) mutation on the variance in triglyceride levels and the absence of associations with total cholesterol levels, are in agreement with this predicted effect.

Hamsten and co-workers (24) estimated in families for serum triglyceride concentration that the genetic (0.33) and cultural, i.e. smoking, alcohol intake and obesity, (0.23) inheritance was of similar significance. In our study, carrier status explains approximately 13% of the total variance in plasma triglycerides indicating that almost half of the genetic heritability is due to this mutation within the three families studied. For HDL cholesterol, carrier status explains a similar effect of the genetic heritability. This reduction of levels of HDL cholesterol was also observed in patients with premature atherosclerosis carrying the LpL(Asn291→Ser) mutation (25).

Sofar, only a few studies were performed on heterozygosity for LpL mutations and the effect of carrier status on the lipid parameters. Wilson et al. (26) found that heterozygotes for the LpL(Gly188→Glu) mutation showed moderate fasting hypertriglyceridaemia only after an age of 40. Secondary factors such as obesity, hyperinsulinaemia and lipid-raising drug use were aggravating factors on the genetic defect. Similar results were found with two Austrian families, carrying the LpL(Gly188→Glu) mutation, in which pronounced postprandial lipidaemia was found (27). In contrast with the LpL(Gly188→Glu) mutation, homozygous carriers of the

LpL(Asn291→Ser) mutation do not develop severe hyperchylomicronaemia (this study,28). Although the heterozygous carriers of both mutations seem to develop hyperlipidaemia in the presence of other predisposing factors, the homozygous carriers do differ in their phenotype. These differences in phenotypic effects of mutations in the LpL gene suggest different underlying mechanisms.

Two studies, in which FCH patients with impaired LpL activity were screened for DNA changes in the LpL gene, showed that only polymorphisms but no mutations causing defective catabolic activity could be found in the LpL gene, implying that LpL is not a significant primary factor leading to FCH (29,30). Recently, Mailly and coworkers (31) showed, among 773 healthy men, that carriers of the LpL(Asp9→Asn) substitution have an significantly higher triglyceride concentration compared with non-carriers. This study suggests that the common LpL(Asp9→Asn) variant is a mutation that has insufficient impact on its own to cause hyperlipidaemia. In combination with other predisposing factors LpL(Asp9→Asn) is associated with the development of hyperlipidaemia. As a consequence, this mutation is also found among healthy individuals but its frequency is lower than in patients with hyperlipidaemia.

Our study showed similar results for the LpL(Asn291→Ser) mutation, association with a significant increase of triglycerides and VLDL-cholesterol and a decrease of HDL-cholesterol in carriers. In addition, this mutation is found with an increased frequency in patients with premature atherosclerosis and FCH (14). Taken together, these findings suggest, that within the families described in this study, the LpL(Asn291→Ser) mutation is one of the predisposing genetic factors to FCH but does not cause the disease on its own. Additional genetic and environmental factors are needed for complete expression of the FCH phenotype.

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Chapter 6

Apolipoprotein E Polymorphism Influences Lipid Phenotypic Expression, but not the Low Density Lipoprotein Subfraction Distribution in Familial Combined Hyperlipidemia

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ABSTRACT

The impact of apo E polymorphism on interindividual variation in plasma lipid, lipoprotein concentrations, and LDL subfraction profiles was studied in 201 well-defined patients (88 men and 103 women) with familial combined hyperlipidemia (FCH). When corrected for the concomitant influences of age, gender and obesity, the allelic variation in the apo E gene was shown to explain a statistically significant portion of the variability in lipid and (apo)lipoprotein concentrations. Carriers of the apo $\varepsilon 2$ allele exhibited a substantial higher plasma triglyceride concentration and a lower low density lipoprotein (LDL) cholesterol level, while subjects with the apo $\varepsilon 4$ allele had significant higher total plasma cholesterol and LDL cholesterol levels. In line with this observation, our FCH population was characterized by an over-representation of the apo E4 allele as compared with a Dutch standard population ($\chi^2=55.2$, $p<0.0001$). The contribution of apo E polymorphism to trait variability was different between sexes for plasma triglyceride, VLDL cholesterol, VLDL triglycerides, and high density lipoprotein (HDL) cholesterol levels. Apo E polymorphism had no impact on chemical composition of VLDL; for LDL particles the apo $\varepsilon 2$ allele was associated with a lower cholesterol to protein (C/P) ratio, whereas the opposite was true for the apo $\varepsilon 4$ allele. Despite the demonstrated impact of apo E polymorphism on plasma lipids and LDL chemical composition, in all phenotypic groups a dense LDL subfraction profile predominated. Thus, apo E polymorphism contributes to the lipid phenotypic expression in FCH, whereas further evidence was obtained that a dense LDL subfraction profile is an integral feature of FCH.

INTRODUCTION

Familial combined hyperlipidemia (FCH) is a common heritable lipid disorder, in which affected individuals show elevations of plasma cholesterol, total triglycerides or both, with differing lipid phenotypes occurring within affected first-degree relatives, together with a high prevalence of premature cardiovascular disease (1-3). Originally, it was supposed that FCH was caused by the variable expression of a single autosomal dominant gene with primary action on plasma triglyceride levels and secondary on cholesterol levels (1). Nowadays, FCH is considered to be more heterogeneous, with on the one hand hepatic VLDL overproduction (4,5), possibly amplified by additional defects as insulin resistance, and on the other hand a deficiency in the clearance of circulating triglyceride-rich lipoproteins (6). To the latter molecular defects in the LpL gene may contribute (7).

Apolipoprotein (apo) E, consisting of 3 common isoforms E2, E3 and E4, is one of the major protein constituents of chylomicrons and VLDL remnants, and is involved in the subsequent catabolism of these particles by allowing specific binding to hepatic receptors. Population studies have shown that the apo E polymorphism could explain a significant part of the inter-individual variability in plasma cholesterol and LDL cholesterol (8-10). In these studies, the apo $\varepsilon 2$ allele was frequently associated with lower levels of total plasma cholesterol, LDL cholesterol and apo B, whereas for the apo $\varepsilon 4$ allele the opposite was observed, inherent to the different binding activities of apo E isoproteins to the hepatic receptor (11-13). Similar effects on lipid and lipoprotein concentrations due to apo E polymorphism were documented in a homogeneous sample of patients with familial hypercholesterolemia exhibiting the same LDL receptor mutation (14). These effects appeared to be dependent of age, gender, and body mass index (BMI), similar to reported epidemiological observations (15,16). Consequently, the effects of apo E polymorphism, age, gender and BMI may, at least in part, also explain the great inter-individual variability in lipid phenotypic expression in FCH, despite the probability of a common defect assumed by its inherited nature.

Therefore, the objective of this study was to estimate the specific impact of apo E polymorphism on plasma lipids, lipoproteins and LDL subfraction profiles in well-defined FCH patients, taking concomitant influences of age, gender and BMI into account.

SUBJECTS AND METHODS

Families

As a part of an ongoing study aiming at the identification of biochemical and

genetical aspects underlying FCH, extended FCH kindreds were recruited by family studies of probands attending the out-patient clinic of the Academic Hospital of Nijmegen. These probands repeatedly exhibited a combined hyperlipidemia with both plasma cholesterol and triglyceride concentrations above the 90th percentile for age and gender. Families were only included when they satisfied the following criteria: the presence of a multiple type hyperlipidemia in first-degree relatives with elevated levels of total plasma cholesterol and/or triglycerides using the age- and sex-related 90th percentile upper levels of the prospective cardiovascular Münster (PROCAM) study (17). Thus, besides a proband presenting a combined hyperlipidemia, the presence of at least one first-degree relative with hypertriglyceridemia or hypercholesterolemia was obligatory. Furthermore, at least one of the first-degree relatives should have cardiovascular disease before the age of 60 years.

Families were excluded when first-degree family members had tendon xanthomata. All individuals were Caucasian with an age of 12 years or older. Information on medical status (i.e., diabetes, renal impairment and liver disease), medication use, alcohol intake, smoking habits, and hormonal status in women was collected by questionnaire. The study protocol was approved by the ethical committee of our Institute. None of the probands was homozygous for the apo $\varepsilon 2$ allele or had hyperlipidemia due to a secondary cause (i.e., diabetes mellitus, hypothyroidism and hepatic or renal impairment), which was excluded by standard laboratory tests. Hypolipidemic drugs were discontinued at least 4 weeks prior to drawing of the blood.

Plasma

Overnight fasted venous blood samples were drawn by vein puncture into K_3EDTA containing vacutainer tubes. Non-local participants were visited at their homes, and blood was transported directly to the laboratory. On arrival at the laboratory within 2 hours, plasma was isolated for determination of the lipid and lipoprotein levels, and the LDL subfraction profile.

Plasma lipid and lipoprotein assays and apo E phenotype determination

VLDL was isolated from whole plasma by ultracentrifugation at density 1.006 g/ml for 16 hours at 36,000 rpm in a fixed angle rotor (TFT 45.6 rotor, Kontron, Zürich), in a Beckman L7-55 ultracentrifuge (Beckman, Palo Alto, USA). High density lipoprotein (HDL) cholesterol was determined by the polyethylene glycol 6000 method (18). LDL cholesterol was calculated by subtraction of VLDL cholesterol and HDL cholesterol from

total plasma cholesterol. For determination of its chemical composition LDL was isolated by seguential ultracentrifugation of the VLDL infranatant for another 20 hours. Total cholesterol and triglyceride concentrations were determined by enzymatic, commercially available reagents (Boehringer-Mannheim, FRG, cat. no. 237574 and Sera Pak, Miles, Italy cat. no. 6639, respectively). To obtain the chemical composition data of VLDL and LDL additional unesterified cholesterol and phospholipids were determined using commercial available reagents (Boehringer-Mannheim, FRG, cat. no. 310328, 691844). The protein content of VLDL and LDL was determined using the Lowry method (19) with chloroform extraction to remove turbidity due to lipids. From these data the mean ratio of cholesterol (unesterified cholesterol plus cholesterol moiety of cholesterol ester [≈ 0.59* weight of cholesterol ester]) to protein was calculated. The chemical composition of each VLDL or LDL particle is expressed in percentage of dry mass of each contributing component (i.e., free cholesterol, cholesterol esters, triglycerides, phospholipids and proteins). Total plasma apo B concentrations were determined by immunonephelometry (20). To achieve accurate results in relation to the Center for Disease Control Standardisation Program, the obtained values were recalculated on the basis of an exchange of sera with Dr. S Marcovina (Northwest Lipid Research Laboratory, Seattle WA, USA). Apo E phenotypes were determined after iso-electric focusing of VLDL lipoproteins, as described previously (21). Results obtained for 534 samples agreed with two exceptions well in comparison to apo E genotyping according to Hixson et al (22) (performed by Dr. M.J.V. Hoffer, MGC-Department of Human Genetics, Leiden University, Leiden; data not shown).

Low density lipoprotein subfractionation

LDL subfractions were detected by single spin density gradient ultracentrifugation (23). After ultracentrifugation up to five LDL subfractions could be distinguished as distinct bands in the middle of the tube concentrated in the following density ranges: LDL1 (1.030-1.033g/ml), LDL2 (1.033-1.040 g/ml), LDL3 (1.040-1.045 g/ml), LDL4 (1.045-1.049 g/ml), and LDL5 (1.049-1.054 g/ml). The ultracentrifugation tubes, containing the LDL subfractions stained with Coomassie Brilliant Blue R, were placed in a specially designed rack and photographed. Accurate documentation of the different LDL subfraction patterns was obtained by scanning the obtained slides in triplicate on a LKB 2202 ultrascan laser densitometer (Pharmacia LKB, Uppsala, Sweden). The mean peak heights (h1-h5) of the LDL subfractions (LDL1-LDL5) on the three scans were used to calculate the variable K as a continuous variable, that best describes each individual LDL

subfraction pattern (24). The contribution of each LDL subfraction, expressed by its peak height (%h1-%h5) relative to the total LDL subfraction profile, (total LDL [100%] = %h1+%h2+%h3+%h4+%h5), was calculated. The relative peak heights of LDL3 and the less frequently occurring LDL4 and/or LDL5 were added to give %h3'=(%h3+%h4-+%h5), where LDL[100%] = LDL1[%h1] + LDL2[%h2] + LDL3[%h3']. When a subfraction profile was characterized by a predominance of buoyant LDL particles (h1-h3>0), variable K was calculated by K=(%h1-%h3')/(%h2-%h3'+1). In case of a predominance of heavy, dense LDL subfractions (h1-h3<0), variable K was calculated by: K=(%h1-%h3')/(%h2-%h1+1). A negative value (-1<K<0) reflects a dense LDL subfraction profile and a positive K value (0≤K<1) a buoyant profile.

Statistical methods

All statistical analyses were performed for affected FCH relatives as a group and because of different frequency distributions in men and women for plasma lipids, lipoproteins, and concomitant effects (i.e., age and BMI), shown by epidemiological studies (15,25), also an additional analysis was performed for men and women separately. Differences between men and women for the quantitative trait means depicted in Table 1 were evaluated by Student's τ test. Allele frequencies were determined by the gene-counting method under the hypothesis of a Hardy-Weinberg equilibrium. Differences in apo E phenotype frequencies between FCH relatives and either spouses or a representative sample of the Dutch population (8) were evaluated by the χ^2 test. Multiple regression analysis was used to estimate on the parameters the possible concomitant effects of age, gender, BMI, smoking habits, and hormonal status in women. Initially, affected FCH subjects were distinguished from unaffected relatives on the basis of elevated lipid concentrations (total plasma cholesterol ≥6.5 mmol/l and/or plasma triglycerides ≥2.0 mmol/l). By this classification age, gender and BMI significantly contributed to the variation of a majority of the parameters, whereas smoking habits and hormonal status in women did not. When percentile data (total plasma cholesterol and/or total plasma triglycerides >90th percentile for age and gender) were used to identify affected relatives, age and BMI did not contribute anymore to the variation in the parameters, since these concomitant effects appeared to be incorporated in the percentile data itself. Therefore, the actually presented analyses were performed with data of relatives classified by using the percentile data. A two-way ANOVA with independent variables 'apo E phenotype' and 'sex' towards dependent lipid parameters followed by Scheffe's post-hoc multiple comparison test was performed to determine the effects of apo E polymorphism on plasma

lipids, (apo)lipoproteins, the chemical composition of VLDL and LDL and the LDL subfraction profile, for each sex separately. Statistical analyses were performed by the Department of Medical Statistics using procedures available in the Statistical Analysis System software package (SAS Institute Inc., Cary, N.C.). A trait difference with a p-value less than 0.05 was considered to be significant.

RESULTS

Subjects

In total, 40 multi-generational FCH kindreds including 660 individuals (558 FCH relatives and 102 spouses) were used in this study. Based on percentile data, 201 individuals were found to be affected by exhibiting a total plasma cholesterol and/or a plasma triglyceride concentration above the 90th percentile for age and gender. The anthropometric measurements of the affected subjects, as well as the plasma lipid and lipoprotein levels (88 men and 113 women) are shown in Table 1.

Table 1: Description of independent and dependent measures in the affected FCH subjects

	Women (n=113)	Men (n=88)	p-value
Independent:			
Age (years)	47.3 ± 16.8	45.1 ± 15.5	n.s.
BMI (kg/m^2)	26.3 ± 3.9	26.7 ± 3.2	n.s.
Dependent:			
Total cholesterol*	6.91 ± 1.35	7.12 ± 1.25	n.s.
Triglycerides*	2.75 ± 1.75	4.03 ± 3.16	0.001
VLDL-cholesterol*	1.19 ± 0.93	1.85 ± 1.44	<0.001
VLDL-triglycerides*	1.86 ± 1.46	3.04 ± 2.70	<0.001
HDL-cholesterol*	1.14 ± 0.34	0.90 ± 0.26	<0.001
LDL-cholesterol*	4.58 ± 1.26	4.39 ± 1.33	n.s.
Apolipoprotein-B (mg/dl)	168.8 ± 35.3	181.1 ± 38.7	0.04
K-value	-0.41 ± 0.27	-0.51 ± 0.24	0.001

Values are presented as mean ± S.D.; n.s. not significant, *in mmol/l.

Both in men as well as in women mean age and BMI were similar. Men exhibited higher concentrations of plasma triglycerides, VLDL cholesterol, VLDL triglycerides and apo B, and lower concentrations of HDL cholesterol. Total plasma cholesterol and LDL cholesterol concentrations were not different between both sexes. Despite lower triglyceride levels in women, both sexes had on the average a substantial negative K-value, which represents a LDL subfraction profile in which small dense LDL particles predominate. In men, this feature was more pronounced.

Phenotype and allele frequencies

Table 2 depicts the phenotype and allele frequencies in our sample of Dutch FCH patients and unaffected relatives compared to a standard Dutch control population (8). Apo E phenotype and apo ε allele frequencies in spouses ($n=102$) were similar compared to the Dutch control population, but differed significantly from those in affected and unaffected FCH relatives (Table 2). The apo $\varepsilon 2$ allele frequency was comparable in both FCH relatives and controls, but the apo $\varepsilon 4$ allele frequency in FCH relatives, especially in FCH patients, was higher.

Effects of apo E polymorphism on lipids and (apo)lipoproteins

The impact of apo E polymorphism on trait means in the 88 male and 113 female affected FCH relatives is summarized in Table 3. Reported average alcohol intake and smoking habits appeared to be similar among the apo E phenotype groups. Of all 558 FCH relatives 6 individuals had an apo E2/2 phenotype without exhibiting a hyperlipidemia. With the whole group of affected relatives differences related to gender were observed for plasma triglycerides, VLDL cholesterol, VLDL triglycerides and HDL cholesterol. The significance of the overall difference for the traits between the 5 apo E phenotypes is depicted by the p-value in Table 3. When phenotype means were compared mutually for both sexes, significant differences between the phenotypes could be observed for plasma triglycerides (phenotype E3/2 vs. E4/4, Scheffé's test $p<0.05$), VLDL triglycerides (phenotype E3/2 vs. E4/4, Scheffé's test $p<0.05$) and LDL cholesterol (phenotype E3/2 vs. E3/3 and E4/4 Scheffé's test $p<0.001$, and vs. E4/3 and E4/2 Scheffé's test $p<0.05$). Within this sample, specific differences between apo E phenotypes for total plasma cholesterol concentration could not be distinguished.

Table 2: Apo E phenotype and allele frequencies in our FCH population (affected and unaffected subjects), compared to two control populations.

Apo E Phenotypes	Standard Dutch control population[A] (n=2018) n (%)	All FCH relatives (n=558) n (%)	Affected (n=201) n (%)	Unaffected relatives (n=357) n (%)	Spouses[B] (n=102) n (%)
E2/2	13 (0.01)	7 (0.01)	0 (0.00)	6 (0.02)	1 (0.01)
E3/2	261 (0.13)	66 (0.12)	27 (0.13)	39 (0.11)	13 (0.13)
E3/3	1128 (0.56)	257 (0.46)	75 (0.37)	180 (0.50)	57 (0.56)
E4/2	45 (0.02)	22 (0.04)	10 (0.05)	13 (0.04)	4 (0.04)
E4/3	512 (0.25)	165 (0.30)	67 (0.33)	100 (0.28)	24 (0.23)
E4/4	59 (0.03)	41 (0.07)	22 (0.11)	19 (0.05)	3 (0.03)
Alleles					
ε2	332 (0.08)	102 (0.09)	37 (0 09)	64 (0.09)	19 (0.09)
ε3	3029 (0.75)	745 (0.67)	244 (0.61)	499 (0.70)	109 (0.74)
ε4	675 (0.17)	269 (0.24)	121 (0.30)	151 (0 21)	27 (0.17)
		χ^2=35.1	χ^2=47.1	χ^2=9.4	χ^2=0.31
		df=2	df=2	df=2	df=2
		p<0.0001	p<0.0001	p=0.009	n.s.

[A]The apo E phenotype and allele frequencies in 2018 randomly selected 35-year-old Dutch males (8); [B]Represents the apo E phenotype and allele frequencies in the spouses of FCH relatives, Differences between different population samples were analyzed by χ^2 analysis; n.s. not significant.

Effects of apo E polymorphism on VLDL and LDL composition

Apo E polymorphism had no detectable effect on VLDL chemical composition (data not shown), except that men exhibited an overall lower protein content, and therefore a higher cholesterol to protein (C/P) ratio in all phenotype groups (data not shown), in agreement with the slightly lower K-values in the males. On the contrary, apo E polymorphism had a significant effect on chemical composition data of LDL particles (Table 4). The relative cholesterol ester content was lower in subjects with phenotype E3/2 compared with those with E4/3 (Scheffé's test $p<0.05$). The same was true when compared with subjects with E4/4 (Scheffé's test $p<0.01$). The triglyceride content was

higher in individuals with phenotype E3/2 compared with those with E3/3 and E4/3 (Scheffé's test $p<0.01$). The phospholipid content was significantly lower in subjects with phenotype E4/4 compared with those with E3/3 (Scheffé's test $p<0.01$) (Table 4).

Table 3: Plasma concentrations of lipids, lipoproteins and apolipoproteins according to apolipoprotein E phenotypes in 201 affected subjects of a familial combined hyperlipidemia sample

Trait	Sex	Apolipoprotein E phenotypes					
		E3/2	E3/3	E4/2	E4/3	E4/4	p-value[A]
Number	♀	15	40	6	41	11	
	♂	12	35	4	26	11	
TC	♀	5 88±1 27	7 10±1 38	6 51±0 81	6 99±1 36	7 51±0 84	0 025
	♂	7 15±2 13	7 32±0 89	6 00±0 82	6 77±0 92	7 30±1 34	
TG	♀	3 49±2 34	2 88±2 05	2 89±2 08	2 59±1 13	1 84±0 93[C]	0 009
	♂‡	6 13±4 29	3 49±3 25	4 08±2 50	4 05±2 97	2 70±1 74[C]	
VLDL-C	♀	1 25±0 82	1 30±1 09	1 43±1 18	1 14±0 83	0 76±0 58	n s
	♂‡	2 84±2 20	1 55±1 34	1 83±1 12	1 75±1 19	1 42±0 93	
VLDL-TG	♀	2 37±1 97	2 03±1 97	2 13±1 77	1 66±0 95	1 15±0 84[C]	0 009
	♂‡	4 97±3 83	2 58±2 84	3 10±1 97	3 04±2 37	1 92±1 21[C]	
HDL-C	♀	1 15±0 40	1 14±0 40	1 04±0 38	1 11±0 25	1 27±0 30	n s
	♂‡	0 91±0 38	0 96±0 22	0 86±0 19	0 89±0 21	0 85±0 36	
LDL-C	♀	3 49±1 08[DD]	4 66±1 12	4 01±1 47[F]	4 74±1 25[F]	5 49±1 04[CC]	<0 001
	♂	3 40±1 24[DD]	4 79±1 22	3 31±0 45[F]	4 14±1 09[E]	5 10±1 56[CC]	
LDL TG	♀	0 50±0 16	0 44±0 12	0 36±0 19	0 48±0 14	0 43±0 12	n s
	♂	0 52±0 21	0 46±0 17	0 35±0 11	0 44±0 12	0 59±0 25	
Apo-B	♀	161 2±55 4	169 7±31 9	151 6±31 8	174 1±33 7	168 0±19 6	n s
	♂	187 0±41 0	176 7±35 6	168 2±16 7	172 5±33 4	193 4±33 6	

Values are presented as mean±SD in mmol/l for lipids and lipoproteins and in mg/l for apoprotein-B. TC total plasma cholesterol, TG total plasma triglycerides, [A]Differences between 5 phenotypes if both men and women are considered together, ‡ sex difference $p<0.01$ Scheffé's post hoc tests [C]$p<0.05$, [CC]$p<0.001$ E3/2 vs E4/4 [DD]$p<0.001$ E3/2 vs E3/3, [E]$p<0.05$ E3/2 vs E4/3, [F]$p<0.05$ E3/2 vs E4/3, n s not significant

A sex difference was observed for the free cholesterol content and for the cholesterol to protein (C/P) ratio. This C/P ratio was lower in subjects with E3/2 compared with

subjects with E3/3 (Scheffé's test $p<0.05$) and higher in subjects with E4/4 compared with those with E3/2 (Table 4).

Table 4: Chemical composition of low density lipoproteins (in percentage of dry mass) according to apolipoprotein E phenotypes in 201 affected subjects with familial combined hyperlipidemia.

Trait	Sex	Apolipoprotein E phenotypes					
		E3/2	E3/3	E4/2	E4/3	E4/4	p-value[A]
%FC	♀	7 8±1 4	7 9±1 5	8 8±1 9	7 9±0 9	8 6±0 8	n s
	♂‡	7 0±1 8	7 6±1 2	6 6±0 5	7 3±1 0	8 0±1 6	
%CE	♀	35 6±5 6	37 2±8 6	41 7±6 3	40 2±3 9[D]	43 4±4 0[EE]	0 001
	♂	33 7±8 0	40 3±4 5	38 8±3 1	39 9±4 6[D]	41 3±7 9[FE]	
%TG	♀	10 4±3 5[CC]	8 1±3 0	7 4±2 6	8 3±2 4[DD]	7 3 ±2 8	0 002
	♂	14 2±7 9[CC]	8 5±5 5	8 3±3 2	8 5±3 4[DD]	9 8±5 5	
%PL	♀	20 8±1 4	20 4±3 3	16 0±7 2	18 3±5 2	17 1±6 4[FF]	0 002
	♂	18 4±6 6	20 1±2 0	18 6±0 7	17 7±4 8	14 2±8 4[FF]	
%PROT	♀	25 4±3 3	26 5±7 0	26 2±4 6	25 3±2 4	23 8±2 2	n s
	♂	26 7±2 7	23 5±2 4	27 8±0 2	26 6±2 4	26 7±3 3	
C/P ratio	♀	1 17±0 26[C]	1 22±0 31	1 32±0 30	1 27±0 16	1 45±0 15[I]	0 009
	♂‡	1 04±0 29[C]	1 37±0 27	1 07±0 08	1 18±0 20	1 23±0 23[E]	

Values are presented as mean±SD, FC free cholesterol, CE cholesterol ester, TG triglycerides, PL phospholipids, PROT proteins, C/P ratio, the ratio of cholesterol (i e , FC plus cholesterol moiety of CE [=0 59*weight of CE]) to protein, [A]Differences between 5 phenotypes if both men and women are considered together, ‡ sex difference $p<0$ 05 - Scheffé's post hoc tests [C]$p<0$ 05, [CC]$p<0$ 01 E3/2 vs E3/3, [D]$p<0$ 05, [DD]$p<0$ 01 E3/2 vs F4/3,[I]$p<0$ 05, [EE]$p<0$ 01 E3/2 vs E4/4,[FF]$p<0$ 01 E3/3 vs E4/4, n s not significant

Effects of apo E polymorphism on LDL subfraction distribution

The distribution of LDL subfractions in a LDL subfraction profile were both described by the amount of cholesterol measured in each separated LDL subfraction (Figure 1), and by the continuous variable K, taking the relative contribution of each LDL subfraction into account (Table 5). Data of 12 affected subjects were missing due to technical errors or lack of sufficient amount of plasma. As a consequence of the effect of apo E polymorphism on total plasma LDL cholesterol, highest individual LDL subfraction cholesterol concentrations were observed in patients with the apo E4/4 phenotype and lowest with apo E3/2 phenotype (Figure 1). However, no effect of apo E polymorphism

on the distribution of LDL subfractions pattern was observed, despite the established quantitative effect on lipid and (apo)lipoprotein concentrations. Affected FCH relatives exhibited a predominance of heavy LDL particles as described by negative K values in all apo E phenotype groups (Table 5). Men showed more dense LDL subfraction profiles (i.e., more negative K values) than women.

Table 5: Distribution of low density lipoprotein subfraction profiles, described by a continuous variable K according to apolipoprotein E phenotypes in 189 affected subjects of a sample of Dutch familial combined hyperlipidemia families.

		Apolipoprotein E phenotypes					
Trait	Sex	E3/2	E3/3	E4/2	E4/3	E4/4	p-value^
Number	♀	15	39	5	40	11	
	♂	12	31	3	23	10	
K-value	♀	-0 34±0 06	-0 44±0 05	-0 36±0 13	-0 46±0 04	-0 25±0 08	ns
	♂‡	-0 45±0 07	-0 52±0 05	-0 67±0 07	-0 52±0 04	-0 45±0 12	

Values are presented as mean±SEM; ^Differences between 5 phenotypes if both men and women are considered together; ‡ sex difference p=0.004.

Additionally, each apo E phenotype showed a predominance of dense LDL3 particles, as measured by the cholesterol content in the subfractions both for men and women (Figure 1). In the general picture there was one exception in women with apo E4/4. Only in this group the distribution of LDL subfractions showed a buoyant profile with highest concentration in LDL1 (Figure 1).

DISCUSSION

Although the genetic nature of FCH seems obvious, it remains to be established whether different affected subjects represent the same metabolic defect or whether additional genetic factors (e.g., gender and apo E polymorphism), or concomitant influences of age and BMI could amplify the lipid phenotypic expression of FCH.

Population based studies have documented the genetic variance of plasma lipids both in normolipidemic subjects (8-10,12) and in hypercholesterolemic subjects (14), associated with the apo E phenotype. In the present study, we could demonstrate that the apo E polymorphism also contributes to the magnitude of lipid and lipoprotein concentrations among affected subjects of 40 well-defined FCH families.

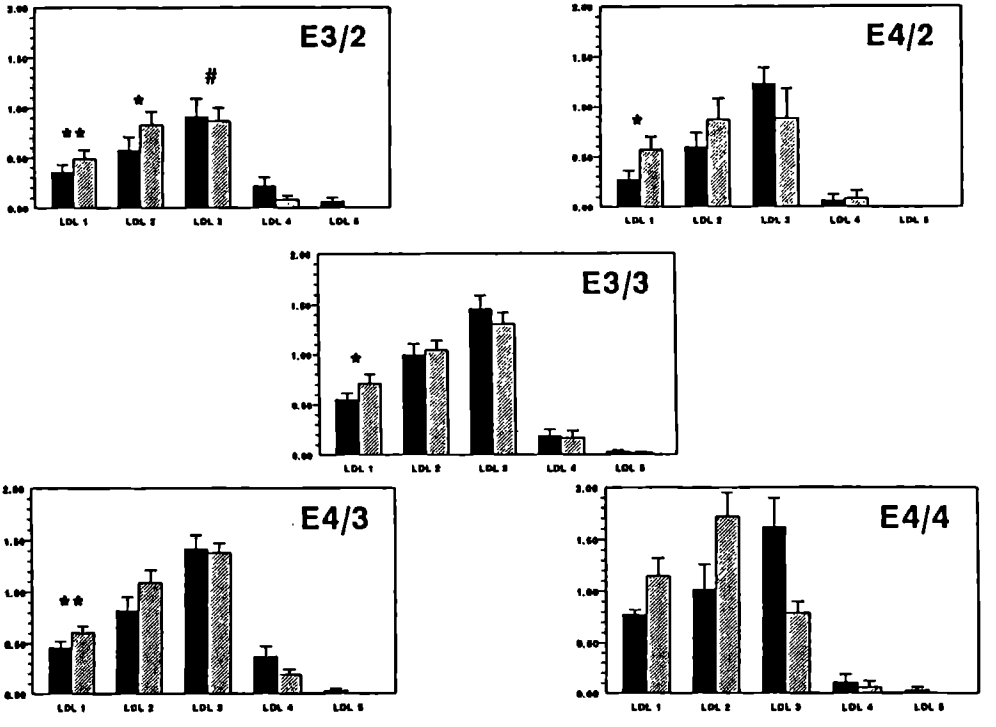

Figure 1: Cholesterol content of 5 isolated LDL subfractions contributing to the total LDL subfraction profile according to the apolipoprotein E phenotypes of 189 affected FCH patients. The black and the hatched bars represent men and women, respectively. Mean sex-difference if all apo E phenotypes are considered together for LDL1 $p=0.006$, for LDL2 $p=0.016$, for LDL3 $p=0.026$, for LDL4 n.s., and for LDL5 n.s.; *$p<0.05$, **$p<0.01$ when equivalent LDL subfractions are compared with E4/4, #$p<0.05$ E3/2 vs. E3/3 or E4/3; all differences are observed both for men and women.

Like familial dysbetalipoproteinemia (FD) in which other factors next to the apo E2/2 phenotype contribute to the expression of the hyperlipidemia (26), a similar coincidence of factors may appear in FCH. Clearly, the presumed overproduction of VLDL particles as a primary cause of FCH, would be exaggerated by the differential effect of the apo ε2 allele on VLDL remnants and of the apo ε4 allele on LDL cholesterol. The effect of the apo ε2 allele was confirmed by the observed accumulation of beta-VLDL in FCH subjects with extensive premature atherosclerosis related to a heterozygote apo E3/2 pattern (27), which was independent of the LDL receptor status as reported by others (28). In our FCH sample, indeed, higher VLDL cholesterol and triglycerides concentrations were observed in subjects with apo E3/2. The observed elevated LDL levels in subjects with the possession of an apo E4 allele hypothetically results from the more efficient uptake of chylomicrons and VLDL remnants by the liver increasing intracellular cholesterol, thereby reducing LDL receptor activity and thus elevating plasma LDL cholesterol levels (11).

When apo E alleles effects are primarily related to FCH, the allele frequency would deviated from normal. In a comparative study between hypercholesterolemic patients and non-obese patients with combined hyperlipidemia (serum cholesterol $\geq$7.5 mmol/l and serum triglyceride $\geq$2.0 mmol/l) the apo ε2 allele, together with smoking habits and fasting insulin concentrations explained 30% of the hypertriglyceridemia and the low HDL-cholesterol levels (29). Besides specific apo ε2 related differences on plasma lipids, also the frequency of the apo ε2 allele was higher in the combined hyperlipidemic group, which confirms that the apo ε2 allele has a plasma triglyceride raising effect in primary hypercholesterolemic patients. We observed, however, a similar allele frequency of apo ε2, but a significant increase in the presence of apo ε4 in our sample of FCH patient as compared with a representative Dutch control population (8) and the spouses in this study. The apo ε2 frequency similar to the representation in the control population is in agreement with the report of Lussier-Cacan et al. confirming the presence of a normal apo E2 frequency in FCH (30). Consequently, this may imply that apo E2 isoform has only secondary modulating effects on a the expression of FCH. The over-representation of apo E4 in our sample corresponds with results in strictly unrelated patients with primary combined hyperlipidemia (31), and also with the frequency found in patients with coronary heart disease (32,33). Moreover, the normal apo E allele distribution in the spouses of our FCH families suggests that the over-representation of apo ε4 is not simply explained by a high apo ε4 allele frequency in spouses. Therefore, Houlston et al. suggested that the possession of the apo E4 allele is one of the predisposing genetic factors

to the development of the combined hyperlipidemic phenotype of FCH (31).

Although affected subjects with hypertriglyceridemia and the apoE3/2 phenotype may exhibit a relative increase of triglyceride in VLDL particles, on average VLDL particles were not affected in their composition by qualitative differences in the apo E isoprotein content. Furthermore, no increase of cholesterol content as seen in so called VLDL remnants could be observed with the apo E 3/2 phenotype. This apparent homogeneity of VLDL particles in FCH comparable with VLDL of normolipidemic subjects has been reported several times (4,34,35). Our results affirm that the produced VLDL particles maintain their normal composition irrespective of the apo E isoprotein component. So, the accumulating effects of apo E2 on VLDL particles may be subordinate to the overproduction. Furthermore, diminished catalytic LpL activity in FCH (6) may prevent increased VLDL remnant formation. The effects of apo E phenotypes on LDL particle composition reflect the effects as observed on plasma lipids. These compositional changes are potentially associated with alterations in LDL heterogeneity (36). A high cholesterol ester content and high C/P ratio is usually associated with large buoyant LDL particles, whereas a high triglyceride content and low C/P ratio coincides with small dense LDL particles (36,37). Only in women with apo E4, exhibiting elevated LDL cholesterol and low plasma triglyceride concentrations the buoyant LDL1 and LDL2 subfractions predominated. Surprisingly, no significant differences in the overall dense LDL subfraction profiles between the various apo E phenotype groups were observed. Thus, the affected FCH subjects in this sample could be distinguished by the presence of a small dense LDL subfraction profile, as reported previously (38-41), despite the documented variation in plasma lipids. We considered several explanations for this apparent contradiction: (i) the compositional differences in LDL due to apo E polymorphism may be too small to cause identifiable changes in LDL subfraction profiles. (ii) it has to be noted that all LDL C/P ratios were below 1.45, which is usually associated with dense particles (36). (iii) pharmacological studies in FCH patients showed the persistence of small dense LDL particles in FCH patients despite substantial reduction of plasma triglyceride concentrations by gemfibrozil administration (39,40). This is in line with the identification of a major gene effect on the small dense LDL subfraction distribution in these FCH families (41).

In conclusion, in the genetically and metabolically heterogeneous lipid disorder FCH we demonstrated that the apo E polymorphism by itself contributes significantly to the lipid and lipoprotein concentrations of affected subjects, independent of its allele frequency. It is likely that in FCH populations the apo $\varepsilon 4$ allele is slightly over-

represented. However, apart from this, apo E polymorphism itself has no distinct effect on the distribution of LDL subfractions. This finding provides further evidence that a LDL subfraction profile consisting of small dense LDL particles is a characteristic feature in FCH patients, irrespective of apo E related impact on plasma lipid and lipoprotein concentrations.

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Chapter 7

The redox status of coenzyme Q10 in total LDL as an indicator of in vivo oxidative modification; studies on subjects with familial combined hyperlipidemia

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ABSTRACT

Familial combined hyperlipidemia (FCH) is characterized by a familial occurence of a multiple-type hyperlipidemia, associated with coronary risk. The latter may be related to increased levels of small dense low-density lipoprotein (LDL) particles which have been found to be more prone to oxidative modification. We isolated total LDL, as freshly as possible from: 12 normolipidemic relatives with a buoyant LDL subfraction profile (group 1), 7 normolipidemics with a dense LDL subfraction profile (group 2), and 16 hyperlipidemic FCH subjects with a dense LDL subfraction profile (group 3). In these non-obese and normotensive men, we studied the resistance of total LDL against Cu^{2+}-oxidation in vitro. In addition, we analyzed the α-tocopherol and the coenzyme Q10 contents of LDL and determined their relation with LDL oxidizability. LDL, isolated from group 3 subjects, was more susceptible to oxidative modification than LDL from group 1 subjects (lag time: 60.4 $\pm$ 8.1 vs. 70.4 $\pm$ 11.4 min; $p<0.05$). For the combined groups, the ratio ubiquinol-10/polyunsaturated fatty acids in LDL together with the basal amount of dienes in LDL, were good predictors of the rate of LDL oxidation ($R^2=0.73$, $p=0.0001$). In both groups 2 and 3, the redox status of coenzyme Q10 (ubiquinol-10/ubiquinone-10) and the ratio ubiquinol-10 to α-tocopherol in LDL were reduced when compared with group 1 ($p<0.05$). The K-value, a measure of the LDL density, correlated with the the redox status ($r=0.37$, $p<0.05$). We conclude that in subjects with FCH total LDL is more prone to oxidation, due to the predominance of dense LDL particles. In addition, the decreased redox status of coenzyme Q10 in LDL from subjects with a dense LDL subfraction profile suggests that the LDL in the circulation is already modified due to lipid peroxidation.

INTRODUCTION

Familial combined hyperlipidemia (FCH), first identified by Goldstein et al. (1), is a genetically and metabolically heterogeneous common lipid disorder, associated with an increased risk for cardiovascular disease (1,2). The trait is characterized by a multiple type hyperlipidemia in first degree relatives. Affected persons exhibit elevated plasma concentrations of cholesterol or triglycerides, or both. In addition, elevated concentrations of apolipoprotein B, and reduced concentrations of high density lipoprotein (HDL) cholesterol are observed. Furthermore, FCH has shown to be associated with a predominance of dense low-density lipoprotein (LDL) subfraction profiles (3,4). Small dense LDL was generally found to be prevalent in patients with coronary heart disease (CAD) (5-10).

Studies on LDL oxidation revealed that small dense LDL particles are more prone to oxidative modification than buoyant LDL (11,12). Oxidation of LDL is supposed to play an important role in early atherosclerosis (13). After being oxidatively modified in the intima, LDL is probably taken up by scavenger receptors of macrophages (14). As a result of uncontrolled uptake of oxidized LDL, macrophages are converted into cholesterol-rich foam cells, a hall-mark of early atherosclerotic lesions (15). Evidence in humans to support the oxidation hypothesis has been provided by studies which showed an association between plasma autoantibodies against oxidatively modified LDL and the progression of cardiovascular disease (16,17). Epitopes of oxidized LDL were also detected in human fatty streaks (18,19).

According to the oxidation hypothesis, LDL is protected against oxidative stress by antioxidants, thereby delaying the formation of modified LDL. The lipophilic antioxidants α-tocopherol, ubiquinol-10, ß-carotene, and lycopene are present in LDL (20). Epidemiological studies suggested that high (>100 mg/day) α-tocopherol intake contributes to reduced risk of atherosclerosis (21,22). Although α-tocopherol is supposed to be the major antioxidant in LDL responsible for the protection against oxidation in vivo (20), extensive work by Stocker and coworkers (23) suggest that the determination of the LDL ubiquinol-10 content is more relevant to assess the initial state of LDL peroxidation in vivo.

Ubiquinol-10 (or reduced coenzyme Q) is an endogenous product of the mevanolate pathway, having coenzymeatic activity in the enzyme system of mitochondria, where it functions as an essential electron carrier in the respiratory system (24). This antioxidant is also present in foods as soy beans, walnuts, almonds, oils, fruits and spinach (25). Despite low concentrations in LDL (0.5-0.8 mol/LDL particle), compared to LDL α-tocopherol

content (8-15 mol/LDL particle), ubiquinol-10 has shown to be the first line defence against oxidative stress in LDL (26). In volunteers, a long term supplementation with Q10 resulted in a two-fold increase in the plasma concentration of ubiquinol-10 (27). This was accompanied by an increased resistance of LDL against oxidation, initiated by aqueous peroxyl radicals, in vitro (27). Beside the contribution of antioxidants, LDL oxidizability in vitro was found to be influenced by the fatty acid composition (28) and the chemical composition of LDL (11).

Although a specific marker to definitively ascertain the diagnosis FCH is still lacking, a recent study shows that a predominance of small dense LDL particles is characteristic for affected FCH relatives (4), especially when accompanied by hyperlipidemia. Regarding the observations on the relatively constancy of the LDL subfraction profile in relation to that of plasma lipid concentrations, especially that of plasma triglycerides (unpublished observations, S.J.H. Bredie, P.N.M. Demacker, A.F.H. Stalenhoef, 1995), we assume that the presence of dense LDL is a helpful metabolic marker for identifying affected relatives of FCH kindreds characterized by variability in plasma concentrations of lipids and cholesterol. Therefore, FCH families offer an unique opportunity to investigate the oxidation characteristics of both dense LDL from subjects with with or without hyperlipidemia and buoyant LDL from normolipidemic subjects, all present in these families.

METHODS

Chemicals

Ubiquinone-10, D,L-α-tocopherol, and butylated hydroxytoluene (BHT) were from Sigma; ubiquinone-10 was reduced to ubiquinol-10, essentially as described by Frei et al. (29). All reagents and HPLC solvents were of high analytical grade.

Subject selection

All participants were selected from 40 well-defined FCH families, consisting of both affected and non affected relatives (4). The diagnosis FCH was based on the following criteria: (i) the presence in first-degree relatives of a multiple type hyperlipidemia with elevated levels of total plasma cholesterol and/or triglycerides using the age- and sex-related 90th percentile upper levels of the prospective cardiovascular Münster (PROCAM) study (30), and (ii) a family history of premature cardiovascular disease before the age of 60 years. Families were excluded when first-degree family members had tendon xanthomata. None of the probands was homozygous for the apo E2 allele, and for all

probands, a secondary cause (i.e., diabetes mellitus, hypothyroidism and hepatic or renal impairment) for the presence of the hyperlipidemia could be excluded by standard laboratory tests.

On basis of the density of the LDL subfraction profiles and plasma lipid concentrations, 35 subjects from 13 families were selected to participate in this study. Twelve subjects were characterized by a buoyant LDL subfraction profile, and 23 subjects by a dense LDL subfraction profile. The method of LDL subfractionation is described elsewhere in this paper. All subjects with a buoyant LDL subfraction profile had normal lipids. From the subjects with a dense LDL subfraction profile, 7 subjects were normolipidemic and 16 subjects were hyperlipidemic. This resulted in three groups of subjects; basal characteristics are summarized in Table 1. None of the subjects were on drug treatment or on a special diet, and none of the subjects used vitamin supplements.

Plasma measurements

Fasting blood samples were collected into vacutainer tubes containing K_3-EDTA (1 mg/ml). The tubes were immediately placed on ice in the dark. Thirty non-local participants were visited at their homes. At two hours after blood sampling, plasma was separated from blood cells by centrifugation at 3600 rpm for 8 min at 4°C. Prior to the measurement of α-tocopherol and ubiquinol-10 concentrations in plasma, saccharose as cryopreservative (final concentration 6 mg/ml), and BHT as antioxidant (final concentration 250 μg/ml) were added.

VLDL and intermediate density lipoprotein (IDL) ($d \leq 1.019$ g/ml) were isolated by ultracentrifugation. After removal of VLDL and IDL, cholesterol and triglycerides were measured in the infranatant and in total plasma. HDL was isolated from whole plasma by the polyethylene glycol 6000 method (31). Cholesterol and triglycerides were determined by enzymatic methods (Boehringer-Mannheim, Germany (cat. no. 237574), and Sera Pak, Miles Tournai, Belgium (cat. no. 6669), respectively). LDL cholesterol was calculated by subtraction.

Analysis of low-density lipoprotein subfraction profiles

Each individual LDL subfraction profile was defined by a continous variable K, as described in detail by de Graaf et al. (32). Briefly, LDL subfractions were separated by single spin density gradient ultracentrifugation, according to an earlier described method (33). After ultracentrifugation the LDL subfractions were visible as distinct bands in the middle of the tube. Up to five LDL subfractions could be distinguished. The tubes were

photographed. Accurate documentation of the LDL subfraction distribution was obtained by scanning the slides on a LKB 2202 ultrascan laser densitometer (Pharmacia LKB, Uppsala, Sweden) (32). The relative peak heights of the LDL subfractions on the scans were used to calculate parameter K as a continuous variable, which best describes each individual LDL subfraction profile. A negative value ($K<0$) reflects a more dense subfraction profile, and a positive K value ($K\geq0$) a more buoyant profile (32).

Oxidation of low-density lipoproteins

Plasma isolation was immediately followed by LDL isolation by density gradient ultracentrifugation (40,000 rpm for 18 h at 4°C) using a SW40 rotor (Beckman, Palo Alto, California, USA) (34). After isolation of total LDL, the protein content of LDL was measured by the method of Lowry et al., with chloroform extraction to remove turbidity, using bovine serum albumin as a standard (35). The oxidation experiments were performed as described by Esterbauer et al. (36) as modified by Princen et al. (34). Briefly, the oxidation of LDL (60 μg apolipoprotein/ml) was initiated by the addition of $CuSO_4$ to a final concentration of 18 μM at 37°C. The kinetics of the oxidation of LDL was determined by monitoring the change of the 234-nm diene absorption in a thermostated UV spectrophotometer (Lambda 12, Perkin Elmer GmbH, FRG), equipped with a nine-position automatic sample changer. Each LDL preparation was oxidized twice in two separate oxidation runs on the same day. Every oxidation run was controlled by analyzing one reference LDL, prepared from a pooled plasma stored at -80°C. The interassay coefficients of variation for the oxidation parameters lag time, oxidation rate and the maximal amount of conjugated dienes formed per milligram of protein of the reference LDL amounted to 1.2%, 5%, and 4.7%, respectively ($n=10$). To guarantee a high reproducibility of the oxidation assay, it appeared to be necessary to clean the quartz cuvettes thoroughly after each 3 oxidation runs. For this purpose, cuvettes were immersed into 2% (v/v) Hellmanex (Hellma, Germany, cat no. 329.001) for 30 min under continuous stirring at a hotplate at 80°C. Subsequently, cuvettes were thoroughly washed with deionized water for 15 min, followed by drying in a stream of filtered air.

Determination of α-tocopherol, ubiquinol-10 and ubiquinone-10 in plasma and LDL

To exclude any oxidation of ubiquinol-10, antioxidants were determined in material as freshly as possible. Consequently, plasma separation was immediately followed by LDL isolation by density gradient ultracentrifugation (40,000 rpm for 18 h at 4°C). LDL was isolated by cautious aspiration. Before extraction, BHT was added to the LDL

preparations to a final concentration of 250 μg/ml.

The concentrations of ubiquinone-10 (oxidized form of coenzyme Q10) in plasma and LDL were determined by high performance liquid chromatography (Spectra Physics model 8800, Breda, the Netherlands) with UV detection at 275 nm, sequentially followed by electrochemical detection (Decade, Antec, Leiden, The Netherlands) for the determination of α-tocopherol and ubiquinol-10 (37). In each run, samples obtained of group 2 and/or group 3 subjects were blindly analyzed together with samples obtained of group 1 subjects. Deoxygenated and transition-metal-free aquous solvents were used. All treatments were performed on ice, in the dark and under nitrogen.

Immediately after isolation, plasma or LDL (200 μl) were mixed with 2.0 ml of ice-cold methanol. Subsequently, 4.0 ml of ice-cold n-hexane was added and the mixture was vortex-mixed for 2 min. To exclude artefacts due to instability, samples were extracted in series of maximal 10. After centrifugation for 2 min at 3600 rpm at 4°C, the hexane upper layer was collected and the extraction procedure was repeated. Both hexane layers were pooled and dried under a flow of nitrogen within 45 min at room temperature. The residue was stored at -20°C until injection within 4 h. Just before injection onto the HPLC column, the residue was dissolved in the mobile phase, consisting of 22.5% (v/v) methanol and 77.5% (v/v) ethanol/isopropanol (95:5) with 20 mM lithiumperchlorate as electrolyte. Twenty μl of sample were injected onto an Inertsil ODS-2 column (200 x 3.0 mm; 5-μm particle size) equipped with a reversed phase guard column (10 x 2 mm) (both from Chrompack, Middelburg, the Netherlands). α-Tocopherol and ubiquinol-10 were eluted isocratically with the mobile phase at a flow rate of 0.35 ml/min. The eluate was monitored with an electrochemical detector. A VT-03 flow cell with GC working electrode (Antec, Leiden, the Netherlands) was used. The applied potential was 600 mV (vs. Ag/AgCl in saturated LiCl). The interassay coefficients of variation for the determination of plasma and LDL concentrations of α-tocopherol were 1.3% and 4.5% (n=6), respectively; of ubiquinol-10: 5.9 and 4.4% (n=8), respectively, and of ubiquinone-10: 3.1% and 5.6% (n=8), respectively.

Stability of ubiquinol-10

Because of the suggested instability of ubiquinol-10 in plasma and especially in LDL (26,38) we performed stability experiments to warrant the validity of our results. In whole blood, ubiquinol-10 was found to be stable for at least 4 h in the dark at 4°C (99.6±3.1% to initial concentrations (n=6)). Ubiquinol-10 concentrations in plasma were stable during storage in presence of 250 μg BHT/ml for up to 1 week at -80°C (98.0±3.0% to initial

concentrations (n=3)). In order to use similar LDL preparations both for the oxidation and antioxidant experiments we isolated LDL by density gradient ultracentrifugation in the absence of BHT. Ubiquinol-10 concentrations in LDL were 104.1 ± 4.6% (n=8) to those concentrations measured in LDL isolated in the presence of BHT. Prior to all extractions we added 250 μg BHT/ml. Ubiquinol-10 concentrations in LDL, however, were similar to those received at extraction without BHT (99.0 ± 2.1% (n=3) vs. BHT method). Until extraction of the LDL preparations, LDL was stored in 2 ml of methanol at -20°C after mixing. Under these conditions, ubiquinol-10 was found to be stable for at least 24 h (100.4±3.8% to initial concentrations (n=6)). Consequently, ubiquinon-10 concentrations during the described storage conditions were stable. After the lipid extraction the combined hexane layers were evaporated under N_2(g) and residues were stored at -20°C until analysis within 4 h. During this time, ubiquinol-10 concentrations were stable (98.0 ±2.0% to initial concentrations (n=6)). However, storage of the lipid residues for 24 h at -20°C resulted in a 43.5±8.7% loss of the ubiquinol-content. Consequently, the ubiquinone-10 content was increased.

Determination of fatty acids in LDL

The concentrations of polyunsaturated (C18:2; C20:4) and of monounsaturated (C18:1) fatty acids in LDL were determined essentially as described in detail by de Graaf et al. (11).

Statistical analysis

Prior to statistical testing, plasma TG and VLDL-TG were transformed logarithmically because of skewing of the distributions. Differences in smoking and drinking habit, and personal history of coronary artery disease was calculated by a chi-square test. A oneway analysis of variance (ANOVA) was used to analyze the differences in the studied parameters between the three groups, followed by additional Tukey's multiple comparison tests. All values are presented as mean ± S.D. Associations between variables were calculated with Pearson's correlation coefficients. All statistical analyses, including logistic regression analysis, were performed using SPSS/PC software (SPSS Inc., Chicago, IL, USA).

RESULTS

Plasma lipids, lipoproteins and K values

Mean concentrations of plasma lipids and lipoproteins and the characterization of the

LDL subfraction profiles of the respective groups are presented in Table 1. With regard to age, body mass index, smoking and drinking habits, groups 1, 2 and 3 did not differ from each other. The personal history of coronary heart disease increased from 0% to 31% in groups 1 to 3, respectively. As compared to groups 1 and 2, group 3 subjects had, by selection, higher concentrations of total cholesterol, LDL cholesterol, VLDL cholesterol, total triglycerides and VLDL triglycerides, lower concentrations of HDL cholesterol, and more negative K values (Table 1).

Table 1: Characteristics of subjects, plasma concentrations of lipids and lipoproteins and the LDL subfraction profile (i.e., K value).

	Group 1 (n=12)	Group 2 (n=7)	Group 3 (n=16)
Age (yr)	41.4 ± 12.7	39.4 ± 12.9	46.2 ± 11.8
BMI (kg/m^2)	23.5 ± 2.2	24.8 ± 2.7	25.8 ± 2.8
smokers (>10 cigarettes/day)	16.7%	14.3%	18.8%
personal history of CAD[§]	0%	14.3%	31.2%
total cholesterol (mmol/l)	5 16 ± 0.96	5.59 ± 0.64	6.67 ± 1.34[†]
total triglycerides (mmol/l)	1 30 ± 0.53	1 60 ± 0.26	3.66 ± 1 15[†‡]
VLDL-cholesterol (mmol/l)	0.36 ± 0.17	0.64 ± 0 18	1.51 ± 0 52[†‡]
VLDL-triglycerides (mmol/l)	0.76 ± 0 41	1.01 ± 0.22	2.86 ± 1.05[†‡]
LDL-cholesterol (mmol/l)	3.28 ± 0.82	3.87 ± 0.61	4.25 ± 1.30
HDL-cholesterol (mmol/l)	1.34 ± 0 28	1.04 ± 0.22	0.87 ± 0.14[†]
K value	0.06 ± 0.07	-0.30 ± 0.20[†]	-0.64 ± 0.16[†‡]

Group 1, buoyant LDL subfraction profile, normolipidemic relatives; Group 2, dense LDL subfraction profile, normolipidemic relatives; Group 3, dense LDL subfraction profile, FCH subjects; [§], $p<0.0001$ (chi-square test), [†], $p \leq 0.005$ vs. group 1, [‡], $p \leq 0.005$ vs group 2, BMI, body mass index.

Oxidation characteristics of LDL and LDL antioxidant contents

The mean lag time of LDL for oxidation was shorter in group 3 than in group 1 (Tukey, $p<0.05$) (Table 2). In group 2, an intermediate mean lag time was measured which, probably due to the small group size, did not differ significantly from both other groups. When we considered groups 2 and 3 as one group, on the basis of characterization of the LDL subfraction profile, we observed that the mean lag time of LDL to oxidation was shorter than that in group 1 (Table 3), Exclusion of the smokers in the respective groups did not affect the present results. The lag time of the total LDL to oxidation

correlated with the density of the LDL subfraction profile of the subjects, expressed as the continuous variable K ($r=0.35$, $p<0.05$). The maximal amount of dienes formed per milligram of LDL protein during oxidation of LDL isolated from FCH subjects were lower than the amount of dienes formed in LDL from group 1. Oxidation rates of LDL and basal amounts of dienes in LDL were similar in the three studied groups (Table 2).

Table 2: Oxidation characteristics of LDL isolated from normolipidemic and FCH subjects.

	Group 1 (n=12)	Group 2 (n=7)	Group 3 (n=16)
lag time (min)	70.4 ± 11.4	66.6 ± 14.1	60.4 ± 8.1†
oxidation rate (nmol dienes/mg protein/min)	22.5 ± 2.3	23.6 ± 2.0	21.4 ± 2.5
dienes (t=0) (nmol/mg LDL protein)	175 ± 12	178 ± 11	167 ± 12
$dienes_{max}$ (nmol/mg LDL protein)	710 ± 70	738 ± 59	646 ± 61†‡

Group 1, buoyant LDL subfraction profile, normolipidemic relatives; Group 2, dense LDL subfraction profile, normolipidemic subjects; Group 3, dense LDL subfraction profile, FCH subjects; †, $p < 0.05$ vs group 1; ‡, $p < 0.05$ vs group 2 .

We examined whether the oxidation characteristics of LDL could be attributed to differences in the basal α-tocopherol and ubquinol-10 concentrations. Plasma concentrations of ubiquinol-10 were related to plasma concentrations of total cholesterol ($r=0.41$, $p<0.01$), and α-tocopherol ($r=0.41$, $p<0.01$). The absolute and relative concentrations of α-tocopherol, ubiquinol-10 and ubiquinone-10 in plasma and LDL are given in Table 4, while the most important results are presented in Fig. 1. No differences were found between the three groups in the LDL α-tocopherol content, quantitated relatively to cholesterol, apolipoprotein or PUFA (Table 4). While the LDL QH_2-10, quantitated relatively to apolipoprotein and PUFA, tended to be lower in group 2 when compared with group 1 (Tukey, $p=0.07$), the Q10 content was significantly increased (Table 4). The fatty acid composition of LDL was similar in all three groups (Table 4).

Remarkably, for all subjects with a dense LDL subfraction profile, both the LDL QH_2-10 to α-tocopherol ratio and the redox status of coenzyme Q10 in LDL (QH_2-10/Q10 ratio) were lower than the ratios in LDL from subjects having a buoyant LDL subfraction profile (Figure 1). In line with this, the redox status of coenzyme Q10 correlated with the density of the LDL subfraction profile ($r=0.37$, $p<0.05$). Even when groups 2 and 3 are considered as one group, characterized by a dense LDL subfraction profile (n=23), both

ratios were significantly lower than those in group 1 (data not shown).

Table 3: Oxidation characteristics of LDL isolated from normolipidemic and FCH subjects

	Buoyant LDL[1] (n=12)	Dense LDL[2] (n=23)
K value	0 06 ± 0 07	-0 54 ± 0 24†
lag time (min)	70 4 ± 11 4	62 3 ± 10 4‡
nmol QH_2-10/g apolipoprotein	700 ± 138	578 ± 223‡
nmol Q10/g apolipoprotein	191 ± 99	304 ± 144‡
μmol Q10/mol cholesterol	32 9 ± 14 0	52 3 ± 24 0‡
QH_2-10/Q10	3 2 ± 1 0	1 9 ± 1 0‡
QH_2-10/αTOH	0 05 ± 0 01	0 04 ± 0 01†

[1]LDL isolated from group 1 subjects, [2]LDL isolated from group 2 and 3 subjects, α-TOH, α-tocopherol, QH_2-10, ubiquinol-10, Q10, ubiquinone-10, †0 0005 < p < 0 005, ‡0 01 < p < 0 05

Regarding our stability experiments (see Methods section), it is unlikely that the observed differences in the ratios were due to oxidation ex vivo A second indication that the shift in the redox status of coenzyme Q10 was not an artefact appeared from the similarity of the redox ratios in total plasma versus the indicated LDL fraction in the various groups.

In the combined groups, we observed that 53% of the variability in oxidation rate could be predicted by the basal amount of dienes in LDL together with the basal LDL QH_2-10 to LDL PUFA content ($p<0$ 0001)

DISCUSSION

Total LDL in FCH subjects (group 3) was less well protected against copper-mediated oxidation than total LDL from normolipidemic relatives with a buoyant LDL subfraction profile (group 1) Even when the subjects of groups 2 and 3, all characterized by dense LDL subfraction profile, were combined, LDL was more prone to oxidation than LDL of group 1 ($p<0.05$) To our knowledge, this is the first time a reduced oxidation resistance of total LDL, in stead of isolated LDL subfractions, is presented for subjects characterized by a dense LDL subfraction profile Possibly, the conformation of dense LDL particles facilitates the accessibility of copper to the apolipoprotein B100, or the premature exhaustion of antioxidants, thereby explaining the increased susceptibility to oxidation (39).

Table 4: Concentrations of α-tocopherol and ubiquinol-10 in plasma and lipoproteins.

	Group 1 (n=12)	Group 2 (n=7)	Group 3 (n=16)
Plasma			
α-TOH (μM)[1]	27.6 ± 10.3	27.5 ± 5.5	42.5 ± 9.0[†]
mmol α-TOH/mol cholesterol	5.3 ± 1.7	5.0 ± 1.1	6.5 ± 1.4
QH_2-10 (nM)	1035 ± 600	1020 ± 569	1159 ± 479
μmol QH_2-10/mol cholesterol	193 ± 81	180 ± 95	177 ± 69
Q-10 (nM)[1]	350 ± 101	461 ± 161	593 ± 228[†]
μmol Q-10/mol cholesterol[1]	69 ± 32	85 ± 35	91 ± 34[†]
LDL			
mmol α-TOH/mol cholesterol	1.9 ± 0.4	2.1 ± 0.6	2.3 ± 0.5
μmol α-TOH/g apolipoprotein	14.3 ± 3.1	14.5 ± 2.6	15.2 ± 3.3
mmol α-TOH/mol PUFA	7.0 ± 1.6	6.8 ± 2.1	7.5 ± 1.5
μmol QH_2-10/mol cholesterol	95.3 ± 18.0	69.6 ± 38.0	92.2 ± 32.0
nmol QH_2-10/g apolipoprotein	700 ± 138	480 ± 252	621 ± 204
μmol QH_2-10/mol PUFA	342 ± 88	227 ± 88	308 ± 100
μmol Q-10/mol cholesterol[1]	32.9 ± 14	62.7 ± 25[†]	47.7 ± 22
nmol Q-10/g apolipoprotein[1]	191 ± 99	351 ± 136[†]	284 ± 147
PUFA/C18:1	2.9 ± 0.5	3.2 ± 0.4	3.0 ± 0.4
C18:2/C18:1	2.6 ± 0.5	2.8 ± 0.4	2.6 ± 0.3

See Table 1 for definition of groups; [†]$p<0.05$ vs group 1; α-TOH, α-tocopherol; QH_2-10, ubiquinol-10; Q10, ubiquinone-10; PUFA, sum of C18:2 and C20.4.

Quantitatively, α-tocopherol is an important antioxidant in LDL (20). However, in line with observations of other groups with subjects on a normal diet, the susceptibility of LDL to oxidative modification was not related to the α-tocopherol content of LDL (34,40-43). From in vitro oxidation studies, ubiquinol-10 is shown to be an antioxidant of the first line (26). LDL, isolated from normolipidemic subjects with dense LDL tended to have a lower ubiquinol-10 content, relatieve to that of apolipoprotein B100 or PUFA content, than LDL from subjects with a buoyant profile. In addition, the redox status of coenzyme Q10 (ratio of reduced form to oxidized form of coenzyme Q10) was substantially reduced in dense LDL particles, independently whether LDL was isolated from normolipidemic or hyperlipidemic subjects (Figure 1). The oxidation of ubiquinol-10

within LDL particles was reported to be accompanied by the formation of lipid hydroperoxides within LDL (26). Our data on the redox status of coenzyme Q10 and the consequences concerning the initial degree of lipid peroxidation concur with data of Alleva et al. (44). For normolipidemic subjects these authors showed that hydroperoxide concentrations were increased in dense LDL compared to buyoant LDL particles. An increased lipid hydroperoxide content in dense LDL may also explain the observed increased susceptibility of dense LDL to copper-mediated oxidation, in vitro (45). In healthy subjects elevated concentrations of plasma lipid hydroperoxides were measured, in vivo, when the redox status of coenzyme Q10 was reduced (46). Thus, the redox status of coenzyme Q10 in dense LDL appears to be a sensitive marker for oxidative changes which take place in LDL in vivo. By careful analysis, we have shown that the reduced redox status of coenzyme Q10 in groups 2 and 3, versus that in group 1, can not be explained by a higher arteficial oxidation of ubiquinol-10 during the several analysis steps.

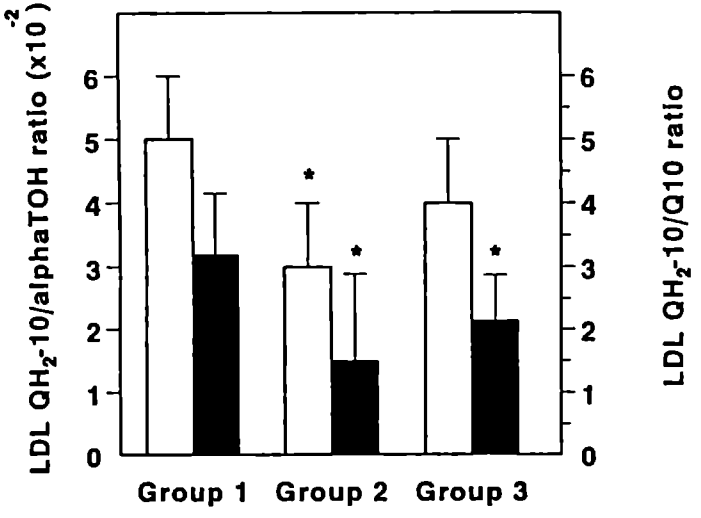

Figure 1: Relative ubiquinol-10 (QH_2-10) concentrations in LDL. LDL QH_2-10 content was quantitated relatively to the α-tocopherol content (left y-axis, open bars), and the ubiquinone-10 content (QH-10, redox status) (right y-axis, filled bars). Groups 1 indicates normolipidemic subjects with a buoyant LDL subfraction profile (n=12); Group 2, normolipidemic subjects with a dense profile (n=7), and Group 3, FCH subjects with a dense profile (n=16); *, $p<0.05$ vs. Group 1.

In this respect it is interesting to note the 3- to 30-fold lower ubiquinol-10 concentration in dense LDL of normolipidemic subjects (38) as compared to concentrations measured in other studies (26,27), and in the present study. We ascribe this to the lengthy isolation procedure of 3 days, including the dialysis step. To prevent loss or oxidation of LDL ubiquinol-10, LDL was not dialyzed in our study. This may explain that we did not find any relation between lag time and ubiquinol-10 content. In agreement with Kontush et al. (47), we found that the basal LDL ubiquinol-10 to PUFA content was negatively correlated with the rate of oxidative modification of LDL. Ubiquinol-10 may indirectly protect the PUFA's against lipid peroxidation, by efficient reduction of α-tocopheroxyl radicals (reviewed in reference 23). In line with results of Stocker and co-workers (26,27) our results stress the promissing role of ubiquinol-10.

Whether a decrease of the redox status of coenzyme Q10 in LDL, due to oxidation of ubiquinol-10, results in minimally oxidized LDL needs to be studied. Compared to native LDL, mildly oxidized LDL exerts important biological effects, at least in vitro. It is believed that these are involved in the early stages of atherosclerosis (48). Minimally oxidized LDL was shown to stimulate the endothelial cell-mediated release of monocyte chemoattractant protein (49) and monocyte colony-stimulating factor (50). On the other hand it may be possible that the radicals which result in oxidation of coenzyme Q10 can also induce tryptophan residue destruction (51). This process occurs in two phases; the earliest phase is independent of α-tocopherol and plays an initial role in LDL lipid peroxidation (51).

In conclusion, dense LDL particles from subjects with FCH are less resistant to oxidation in vitro than buoyant LDL from normolipidemic relatives. Compared with subjects with an overall buoyant LDL subfraction profile, the redox status of coenzyme Q10 was reduced in subjects with an overall dense LDL subfraction profile, independently of the plasma concentration of cholesterol or triglycerides. This suggests that ubiquinol-10 is an important indicator of oxidative modification in vivo. Future investigations to assess oxidative stress in subjects at risk for coronary heart disease should include the redox status of coenzyme Q10.

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Chapter 8

Segregation Analysis of Plasma Apolipoprotein-B Levels in Familial Combined Hyperlipidemia

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ABSTRACT

Familial combined hyperlipidemia (FCH) is a heritable lipid disorder, which is associated with an increased risk of premature cardiovascular disease. An elevated plasma apolipoprotein-B (apoB) concentration is reported to be a diagnostic feature of the disorder. Recently we demonstrated a strong relation between plasma apoB concentrations and the cholesterol concentration in very low density lipoproteins (VLDL) plus low density lipoproteins (LDL), both elevated in FCH families. Therefore, examination of the inheritance of elevated plasma apoB levels in FCH families may reveal important information about the mechanism responsible for the aggregation of elevated plasma lipids in FCH. This study included 663 Dutch family members in 40 families ascertained through FCH-probands. Plasma apoB concentration correlated significantly with apoB-related cholesterol both in the probands and in the relatives ($r=0.83$ and $r=0.90$, respectively). Adjustment for age, gender, BMI and smoking habits accounted for 35.7% of the variation in apoB levels, and there was strong familial aggregation in adjusted apoB levels in these families. Complex segregation analysis was performed to determine the mechanism of inheritance behind this familial aggregation. The aggregation of elevated apoB levels was best explained by a major gene effect inherited by a codominant mechanism. Estimated mean apoB levels for the three supposed genotypes AA, AB and BB were 111.5, 126.7 and 165.7 mg/dl, respectively, with relative frequencies of 43.5%, 44.9% and 11.6%, respectively. In conclusion, despite assumed metabolic and genetic heterogeneity of FCH, there is clear evidence for a single gene effect on apoB concentrations in families ascertained through FCH. Linkage studies based on this analysis may further clarify the molecular basis of the apoB regulation in these families.

INTRODUCTION

Familial combined hyperlipidemia (FCH) is the most common form of heritable lipid disorders, and is associated with cardiovascular disease (1). This multiple phenotype hyperlipidemia is characterized by elevations of the total plasma cholesterol concentration and/or the plasma triglyceride concentration, consequently resulting in the presence of different lipid phenotypes in first-degree relatives. Furthermore, an elevated plasma apolipoprotein-B100 (apoB) concentration, a decreased high density lipoprotein (HDL) concentration, and a preponderance of small dense low density lipoprotein (LDL) particles are observed. Initially, FCH was thought to be inherited as a single gene disorder with a major gene effect on triglyceride levels (1). Recently this was supported by a segregation analysis in 55 British FCH families (2). Other studies, however, have shown FCH to be a more heterogeneous lipid disorder, comprising features of the insulin resistance syndrome, hyperapobetalipoproteinemia and familial dyslipidemic hypertension (3-5), which in turn are all associated with the presence of small dense LDL (6). Still, the absence of a specific metabolic or genetic marker complicates the diagnosis of FCH in individual patients.

In FCH, the hyperlipidemia is caused by hepatic overproduction of very low density lipoprotein (VLDL)-apoB100 (7,8). Consequently, elevated levels of apoB containing lipoproteins (i.e., intermediate density lipoprotein [IDL] and LDL particles) are observed. A delayed clearance of the triglyceride-rich apoB containing lipoproteins, in part caused by impaired lipoprotein lipase activity may modify the lipid phenotypic expression (9,10). Elevated lipid levels in FCH reflect elevated VLDL and LDL cholesterol concentrations which correspond with an increase in respective particle numbers (8,11,12). Since each VLDL and LDL particle always contains one apoB molecule per particle, a strong correlation can be expected in FCH between total plasma apoB and its related cholesterol in VLDL and LDL particles. Based on this, the individual plasma apoB concentration represents the actual VLDL and LDL concentrations, which may show some variability in time in FCH patients (13).

Studies have also shown the association between elevated apoB levels and cardiovascular disease, which is a feature of FCH as well (14-16). Some studies show that the apoB level is a better predictor of premature cardiovascular disease than the plasma lipids are (17,18). Complex segregation analyses demonstrated familial aggregation of elevated apoB levels in families presenting premature cardiovascular disease, in families with familial hyperlipidemia, and in healthy volunteers (19-26), but conclusions about the specific genetic mechanism were contradictory to date. This may be the reason that

linkage studies could not identify conclusively the locus responsible for the elevation of apoB levels (20,27-30), although some gene markers showed some association (31) Before further linkage analyses with candidate genes can be undertaken, it is critical to have a better model of inheritance

In the present study, segregation models were examined to explain the familial clustering of elevated apoB levels measured in individuals from 40 well-defined Dutch FCH families. Single gene effects and polygenic components were tested by using class D regressive models of Bonney et al (32), as implemented in the Statistical Analysis for Genetic Epidemiology (SAGE) package

MATERIALS AND METHODS

Study Population

The FCH pedigrees considered here have been studied for inheritance of LDL subfraction profiles (33) The families were ascertained through probands exhibiting a combined hyperlipidemia with both plasma cholesterol and triglyceride concentrations above the 90th percentile, adjusted for age and gender, as obtained from the Prospective Cardiovascular Munster (PROCAM) Study (34) These values were consistent over several measurements in which the probands had not been given any lipid-lowering drugs Families were included when a multiple type hyperlipidemia with levels of total plasma cholesterol and/or triglycerides above the 90th percentile was present Thus, besides the proband presenting with combined hyperlipidemia, at least one first-degree relative exhibited hypercholesterolemia or hypertriglyceridemia

All probands were tested for an underlying cause of their hyperlipidemia (i e , diabetes mellitus, hypothyroidism and hepatic or renal impairment) The presence of one of these causes excluded them and their families from further analysis None of the probands in these families was homozygous for the apo $\varepsilon 2$ allele and none of the first-degree relatives had tendon xanthomata In addition, to refine the selection procedure, the 95th percentile for plasma cholesterol and triglycerides was used if the body mass index exceeded 30 kg/m^2, or an alcohol consumption of more than two units (one unit=one consumption) per day was present

In total, 40 multi-generational families (2 to 4 generations) were included in this study, containing 40 FCH probands and 623 family members All individuals were Caucasian above the age of 10 years Everyone filled out a questionnaire in order to collect information on medical status, medication use, alcohol intake and smoking habits The study protocol was approved by the ethical committee of the University Hospital of

Nijmegen.

Plasma

After an overnight fast and a withdrawal of lipid-lowering medication for at least four weeks, blood was drawn by venipuncture into EDTA containing vacutainer tubes. Non-local participants were visited at their homes, and blood was transported directly to the laboratory. Plasma was isolated within 3 hours for determination of the lipid and lipoprotein levels.

Measurement of Lipid, Lipoprotein and Apolipoprotein levels

VLDL was isolated from whole plasma by ultracentrifugation at density=1.006 g/ml for 16 hours at 36,000 rpm in a fixed rotor (TFT 45.6 rotor, Kontron, Zurich), in a Beckman L7-55 ultracentrifuge (Beckman, Palo, U.S.A.). High density lipoprotein (HDL) cholesterol was determined by the polyethylene glycol 6000 method (35). LDL cholesterol was calculated by subtraction of VLDL cholesterol and HDL cholesterol from total plasma cholesterol. Respective cholesterol and triglyceride concentrations were determined by enzymatic, commercially available reagents (Boehringer-Mannheim, Germany, cat. No.237574 and Sera Pak, Miles, Italy cat. No. 6639, respectively). Total plasma apoB concentrations were determined by immunonephelometry (36). To achieve accurate results in relation to the Center for Disease Control Standardization Program, obtained values were recalculated on the basis of an exchange of sera with Dr. S. Marcovina (Northwest Lipid Research Laboratory, Seattle WA, U.S.A.).

Statistical Analysis

Pearson correlations coefficients were calculated for the relation between crude plasma apoB values and apoB related cholesterol in VLDL plus LDL. Subsequently, multiple linear regression was used to examine the association between the apoB level and age, gender, BMI, smoking habits, and alcohol intake, as conforming to other reports (19,21,23-26). Based on the results of this analysis, those covariates that significantly influenced apoB level were selected. Given this regression model, predicted apoB levels were calculated and residual values were computed by subtracting each individual's apoB level from their predicted value. These residuals represent the fraction of the apoB level that is not predicted by the selected environmental variables but may represent the genetic contribution to the apoB level. Because these residuals can be positive or negative, the mean apoB level was added back in and this adjusted apoB level was used in further

analysis.

Segregation analysis

To investigate the role of genetic and environmental influences on apoB levels, a series of class D regressive models (32) available in the Statistical Analysis for Genetic Epidemiology (SAGE) program (release 2.2) was used. The models assume that variation among individuals for a quantitative trait is the result of a major gene effect and residual variation which may reflect both familial correlations and individual variation (24). A 'general model' describes the distributions of apoB levels in terms of the independent contribution of a single factor with a major effect, residual familial correlations and non-genetic effects (22). This 'general model' emanates from two alleles at a single locus (denoted A and B) resulting in three possible 'types' of individuals (AA, AB and BB), termed 'ousiotypes' (37). The mean apoB value associated with each 'type' is denoted μ_{AA}, μ_{AB} and μ_{BB}, with a variance σ^2_{AA}, σ^2_{AB}, σ^2_{BB}. The 'ousiotype' effects may be attributable to a genetic or an environmental factor. The parameter p is defined as the frequency of allele A; q= (1-p) equals the frequency of allele B. Founder individuals are assumed to come from a population in Hardy Weinberg equilibrium. Individuals characterized by the 'types' AA, AB and BB are assumed to transmit the allele A with probabilities τ_{AA}, τ_{AB} and τ_{BB}, respectively. These transmission probabilities are used to calculate the probabilities of all three 'types' for individuals whose parents are in the pedigree. In addition, the parameters ρ_{sp}, ρ_{po}, ρ_{mo}, ρ_{fo} and ρ_{ss} denote spouse, parent-offspring, mother-offspring, father-offspring and sib-sib correlations, respectively. In this setting, the parent-offspring correlation provides an estimate of the polygenic heritability, i.e., $h^2=2\rho_{po}*\sigma^2/\sigma^2_T$, where σ^2_t is the total variance and σ^2 is the variance conditional on the restricted model (assuming $\rho_{po}=\rho_{ss}$). Hypotheses were tested by comparing the 'general model' to various submodels, in which certain parameters were restricted to specific values. The 'major environmental model' restricts $\tau_{AA}=\tau_{AB}=\tau_{BB}=p$. In this model the prior probability of an offspring having a certain 'ousiotype' is completely independent of parental 'ousiotypes'. Mendelian models constrain these segregation probabilities τ_{AA}, τ_{AB} and τ_{BB} to be 1.0, 0.5 and 0.0, respectively. The most general Mendelian model is a 'codominant' model where each 'ousiotype' has a separate mean. The 'dominant model' further restricts $\mu_{AA}=\mu_{AB}$, while the recessive model restricts $\mu_{AB}=\mu_{BB}$. Submodels containing ρ_{po}, ρ_{mo}, ρ_{fo} and ρ_{ss} equal to zero are equivalent to restricting the 'polygenic component' to zero. Restricted models are compared to the general model by using the likelihood ratio test (LRT). For non-hierarchical models, Akaike's information criterion

(AIC) was used to compare the fit of the models. For a given model AIC=-2ln(L)+2*x (x=number of parameters estimated in the model). The model with the lowest AIC is considered the most parsimonious model (38).

Segregation analysis was performed on 40 families which included 825 individuals, although 162 individuals had missing date (primarily these included dead family members linking other relatives) In this study no normalizing transformation was performed because analysis of untransformed data is easier to interpret and can be compared more easily to the published studies. In addition, normalizing transformations of a biologically skewed variable may lead to a large reduction in the power to detect a major gene when one exists (39). Families were selected through two affected probands Consequently, the lipid inclusion criteria (i e , both plasma cholesterol- and triglyceride levels) of probands versus non-probands showed large differences (Table 1) Therefore, ascertainment correction was undertaken by conditioning the phenotypes of family members on those of the affected primary probands and that of one extra individual with elevated lipid levels per pedigree (33)

RESULTS

Study population

The study included 663 individuals with an age between 10-88 years old The correlation between crude plasma apoB concentration and apoB related cholesterol concentration (i e., VLDL plus LDL cholesterol), both for the 40 probands plus 40 extra affected subjects of each family and for the remaining relatives is presented in Figure 1

Table 1: Characteristics of the Study Population

	Probands (n = 80)		Family members (n = 583)		Total (n = 663)	
Characteristics	Mean	SD	Mean	SD	Mean	SD
Age (years)	54 6	11 4	40 8	16 6	42 4	16 6
BMI (kg/m^2)	26 7	3 8	24 4	3 7	24 7	3 8
Crude apo-B (mg/dl)[A]	175 8	40 7	127 8	37 0	133 8	40 7
Adjusted apo-B (mg/dl)[B]	156 9	43 1	130 7	29 3	133 8	32 3

[A]Crude apoB levels were missing for 7 probands and 72 family members, [B]Adjusted apoB levels were missing for 13 probands and 78 family members

In both groups, a significant correlation was observed (0.83, $p<0.0001$ and 0.90, $p<0.0001$, respectively). Characteristics of the study population are presented in Table 1. BMI values varied between 13.6 and 40.9 kg/m^2, crude apoB levels between 60 and 298 mg/dl and the adjusted apoB levels between 42.3 and 277.7 mg/dl. Significant differences between probands and family members were seen in mean values of both the crude and the adjusted apoB levels.

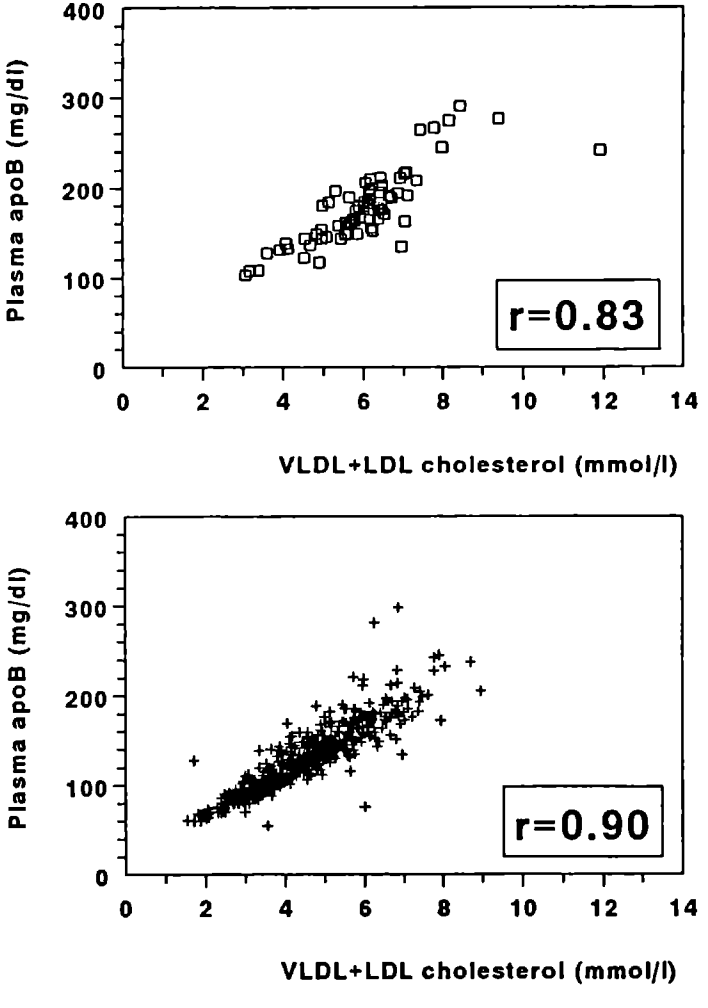

Figure 1: The correlation between plasma apoB and its related cholesterol in VLDL plus LDL fractions in all 80 probands (top) and in the remaining FCH relatives (bottum).

Adjustment of the apoB level

Non-lipid covariates (i.e , age, age^2, gender, BMI, smoking habits [0=not smoking, 1=one or more cigarettes/day] and alcohol consumption [0=no alcohol consumption, 1=one or more units/day] were examined in association with plasma apoB level. Table 2 shows the covariates and their regression coefficients used in the adjustment of the apoB levels. Age^2 and alcohol consumption were excluded from the adjustment procedure because they did not contribute significantly. Although in this study gender was not significantly associated with apoB, it was included in the adjustment procedure to make our results more comparable with other studies. The variables combined explained 35 7% of the variation in apoB levels

Table 2: Regression coefficients (β) and standard error (S E) for the four variables used in a multiple linear regression model to adjust the crude plasma apoB level

Variables	β	S E	P-value
Age (years)	1 05	0 09	0 0001
BMI (kg/m^2)	2 73	0 40	0 0001
Gender (female)	-3 27	2 74	0 2334
Smoking ($\geq$ 1 cigarette/day)	13 55	3 00	0 0001

Segregation Analysis

In a first segregation analysis on the total study population the data were consistent with two different models of inheritance a) a codominant major gene effect explaining 44% of the variance in apoB levels with an insignificant polygenetic component of h^2=1-%, and b) an environmental model explaining 16% of the apoB level variance with a polygenetic component of h^2=38% (data not shown) This ambiguous result may have been caused by either genetic heterogeneity among these 40 pedigrees, yielding a mixture of different mechanisms influencing apoB levels, or by extreme apoB levels in certain families or particular individuals.

In order to evaluate the extent to which each family supported one model over the other, the -2ln(L) scores of both an environmental model (-2ln$[L]_L$) and a codominant model (-2ln$[L]_C$) were calculated for each family. The ratio -2ln(L_I/L_C) measures the extent to which each family favored one or the other model (19,24,40,41) Families which favored an environmental model would hava a -2ln(L_I/L_C) ratio$<$0, and families with a

$-2\ln(L_E/L_C) > 0$ favored a codominant model. As shown in figure 2, the distribution of the likelihood ratio's was centered around zero, suggesting most families cannot discriminate definitively between these models. The procedure separated 19 families more favoring an environmental model and 21 families more favoring a codominant model. One family, however, seemed to give an extreme preference to the environmental model ($-2\ln[L_E/L_C] = -13.5$). This particular family (containing twelve subjects in three generations) included the individual with the highest adjusted apoB level and was excluded from further analysis.

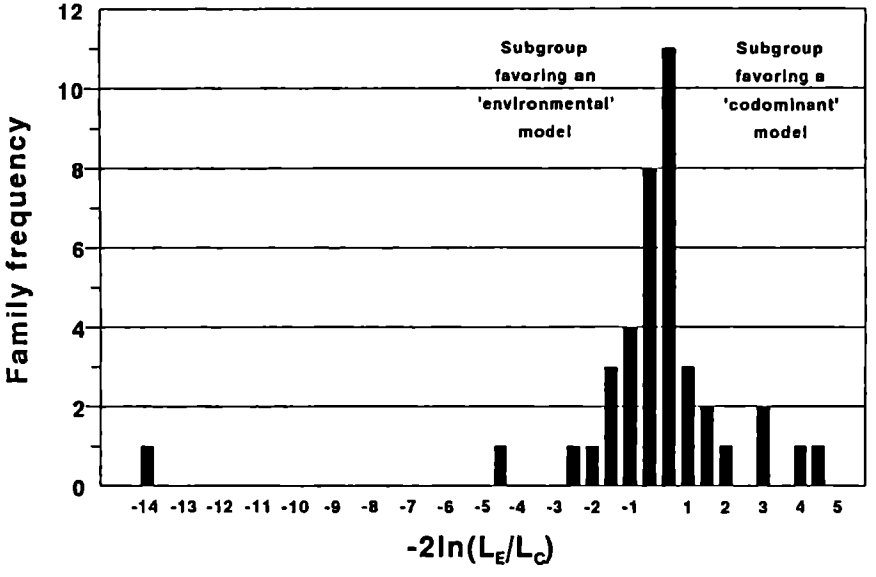

Figure 2: Relative likelihood of the environmental model $-2\ln(L_E)$ and the codominant model $-2\ln(L_C)$. The ratio $-2\ln(L_e/L_c)$ measures the extent to which each family supported one model over the other.

The results of a second series of analyses testing the different genetic models on the 39 remaining pedigrees are shown in Table 3. All models assumed a single correlation among parents and offspring (ρ_{po}), which appeared to be equal to the sib-sib correlation (ρ_{ss}). Therefore, all submodels were assumed to have a single correlation for first-degree relatives ($\rho_{po}=\rho_{ss}$). The best fitting one distribution model (model 4) showed insignificant spouse correlations ($\rho_{sp}=-0.14\pm0.07$) and significant residual familial correlations ($\rho_{po}=\rho_{ss}=0.15\pm0.03$). Using this familial correlation, the polygenetic heritability was

estimated to be $h^2 = 2\rho_{po} * \sigma^2/\sigma^2_T = 0.29$, which is compatible with published estimates from other studies. The non-Mendelian two distribution model (model 6) showed a significant improvement of the -2ln(L). The three distribution non-Mendelian model (model 7) gave no further improvement of the fit. Inclusion of a two distribution Mendelian mechanism (model 9) showed a significant improvement of the -2ln(L), compared with model 4. The two distribution Mendelian models (models 8 and 9) looked very similar to the two distribution non-Mendelian models (models 5 and 6) with $\rho_{sp} = -0.11 \pm 0.10$ and $\rho_{po} = \rho_{ss} = 0.22 \pm 0.05$. Extension of a two distribution Mendelian model (model 9) to a three distribution Mendelian model (model 11) revealed a significant improvement of the -2ln(L). The analysis presented a general model very similar to the best fitting codominant model (model 11). Although the excluded family had a substantial influence on the -2ln(L) used to select the most parsimonious model, no major differences were seen in the parameter estimates. Comparison of the -2ln(L) scores of submodels against the general model (model 12) showed that models 1, 2, 3, 4, 7, 8, and 9 could be strongly rejected ($p < 0.001$), while models 5, 6, and 10 could only be weakly rejected ($p < 0.05$). Only model 11 could not be rejected. Rejected environmental models 5, 6 and 7 did not contradict the most parsimonious model (model 11) because they showed that variation in apoB levels was strongly explained by a genetic component ($\rho_{po} = \rho_{ss} = 0.24 \pm 0.05$ to 0.25 ± 0.05). Comparison of model 5, 6 and 7 to model 11, demonstrated that the significant familial correlation in apoB levels was absorbed by the inclusion of a codominant mechanism of inheritance. Therefore, the segregation of elevated apoB levels in 39 of the 40 FCH families was best explained as the result of a codominant major gene effect.

DISCUSSION

In the present study, a segregation analysis was performed to examine genetic mechanisms that could explain the familial clustering of elevated apoB levels as observed in 663 individuals from 40 well-defined Dutch families. These families were ascertained through FCH, a common hyperlipidemia associated with premature cardiovascular disease. The observed Mendelian mechanism with a codominant mode of inheritance for the segregation of elevated plasma apoB levels in this study agrees very well with the reported inheritance of apoB levels in other populations containing families of patients with coronary artery disease and families with familial dyslipidemic hypertension (20,21,24,25). Such families most probably also contain patients who satisfy the criteria for FCH. On the other hand, the found mechanism of inheritance is not in contradiction

Table 3: Segregation analysis of adjusted plasma apoB levels[@] in 651 subjects of 39 FCH families

Model	p	μ_{AA}	μ_{AB}	μ_{BB}	σ^2	τ_{AA}	τ_{AB}	τ_{BB}	ρ_{sp}	$\rho_{po}=\rho_{m}$	-2ln(L)	AIC	X^2 (df)
1. Sporadic	[1 0]	130 6 ±1 3	$=\mu_{AA}$	$=\mu_{AA}$	821 4 ±52 2			.	[0 0]	[0 0]	4,736 3	4,740 3	68 7 (8)**
2 Sporadic, ρ_{sp}	[1 0]	130 8 ±1 2	$=\mu_{AA}$	$=\mu_{AA}$	814 9 ±51 9				-0 20 ±0 08	[0 0]	4,729 6	4,735 6	62 0 (7)**
3 Sporadic, $\rho_{po}=\rho_{m}$	[1 0]	126 8 ±1 9	$=\mu_{AA}$	$=\mu_{AA}$	807 1 ±53 7				[0 0]	0 17 ±0 04	4,701 1	4,707 1	33 5 (7)**
4 Sporadic, ρ_{sp}, $\rho_{po}=\rho_{m}$	[1 0]	127 7 ±1 8	$=\mu_{AA}$	$=\mu_{AA}$	796 2 ±52 3				-0 14 ±0 07	0 15 ±0 03	4,697 4	4,705 4	29 8 (6)**
5 Environmental, $\rho_{po}=\rho_{m}$	0 86 ±0 04	125 4 ±1 9	$=\mu_{AA}$	$=\mu_{AA}$	201 4 ±13 3	714 4 ±52 5	=p	=p	=p	[0 0]	0 25 ±0 05	4,682 1	4,692 1	14 5 (5)*
6 Environmental, ρ_{sp}, $\rho_{po}=\rho_{m}$	0 85 ±0 04	125 8 ±1 8	$=\mu_{AA}$	$=\mu_{AA}$	196 5 ±13 4	703 3 ±52 7	=p	=p	=p	-0 11 ±0 10	0 24 ±0 05	4,680 9	4,692 9	13 3 (4)*
7 Environmental, ρ_{sp}, $\rho_{po}=\rho_{m}$	0 85 ±0 04	125 8 ±5 4	125 8 ±14 7	$=\mu_{AA}$	196 5 ±13 4	703 4 ±52 7	=p	=p	=p	-0 11 ±0 10	0 24 ±0 05	4,680 9	4,694 9	13 3 (3)**
8 A dominant, $\rho_{po}=\rho_{m}$	0 88 ±0 04	125 2 ±2 0	$=\mu_{AA}$	$=\mu_{AA}$	199 8 ±15 8	724 6 ±56 1	[1 0]	[0 5]	[0 0]	[0 0]	0 24 ±0 04	4,690 9	4,700 9	23 3 (5)**
9 A dominant, ρ_{sp}, $\rho_{po}=\rho_{m}$	0 87 ±0 05	125 6 ±2 0	$=\mu_{AA}$	$=\mu_{AA}$	193 9 ±17 8	718 8 ±59 6	[1 0]	[0 5]	[0 0]	0 11 ±0 10	0 22 ±0 05	4,689 6	4,701 6	22 0 (4)**
10 Codominant, $\rho_{po}=\rho_{m}$	0 67 ±0 06	110 5 ±3 0	127 4 ±3 4	166 0 ±5 1	441 5 ±47 4	[1 0]	[0 5]	[0 0]	[0 0]	(0 0) bnd	4,678 7	4,688 7	11 1 (4)*
11 Codominant, ρ_{sp}, $\rho_{po}=\rho_{m}$	0 66 ±0 06	111 5 ±3 0	126 7 ±3 5	165 7 ±5 2	442 6 ±47 2	[1 0]	[0 5]	[0 0]	-0 20 ±0 10	(0 0) bnd	4,674 7	4,684 7	7 1 (3)
12 General, ρ_{sp}, $\rho_{po}=\rho_{m}$	0 75 ±0 06	110 3 ±3 2	128 6 ±4 5	167 7 ±5 4	413 2 ±61 4	0 91 ±0 05	0 35 ±0 10	0 09 ±0 13	-0 18 ±0 11	0 00 ±0 06	4,667 6	4,687 6	Reference

[@] Apo-B adjusted for: age, gender, body mass index and smoking habits.
[ ] restricted

* = different from the general model, $p<0.05$.
** = different from the general model, $p<0.01$.

with the previous reported major gene effect on plasma triglyceride levels (1,2), because VLDL particles, which carry most of the total triglycerides, show a consistent composition of lipids and apoB protein in FCH (8,11,12).

Total plasma apoB was determined by immunonephelometry and obtained values were adjusted for variation due to age, gender and smoking habits prior to the segregation analyses (19-26). In the present study also BMI was shown to be highly correlated with apoB levels.

Because a first segregation analysis could not discriminate conclusively between two models of inheritance, a more detailed analysis was performed on each family. In contrast with other 39 families, one family seemed to have an extreme preference to a two distribution non-Mendelian model. The exclusion of this single outlying family in a second segregation analysis, only influenced the -2ln(L) scores and through these the selection of the most parsimonious model, whereas estimates of model specific parameters were remarkably consistent. Therefore, this family can be seen as an indication of rare heterogeneity, also observed in other family samples (23-25).

Both our first and second segregation analysis showed a significant familial correlation of $\rho_{po}=\rho_{ss}=0.24$ (model 7), which appeared not to be influenced by the excluded family. This estimated correlation is comparable to that reported by others (0.13 to 0.47) (23,24). Although examined in completely different study populations, most studies reported a codominant model as the most parsimonious fit on their apoB data (20-22,24,25,31). A few studies preferred, however, a dominant or recessive model (19,23,26). The estimated relative frequency of the 'low' apoB allele at 66% is in agreement with other reports (20-22,24,25,31). These consistent findings suggest that genetic control on apoB levels is comparable in subjects with different dislipidemia phenotypes. Some of the remaining differences in the results may be explained by the large differences in ascertainment criteria of the analyzed study populations, since studies ascertained families through probands who suffered from CAD (19,21,25), who were patients of a lipid clinic (22,23), who were submitted to elective coronary angiography (24) or after a coincident detection of CAD in the family (20,31). This study analyzed its sample after correction on ascertainment. Although the influence of ascertainment bias on the segregation analysis is still a subject of debate, inadequate correction for ascertainment bias can provide misleading results, suggesting the presence of a major locus when one is absent (42).

FCH is caused by hepatic overproduction of VLDL-apoB100, subsequently leading to elevated levels of apoB containing IDL and LDL particles, of which the latter are

predominantly smaller and denser compared to normolipidemic subjects (11,14). The finding of a genetic mechanism for elevated apoB levels in this FCH population suggests that there is genetic control of hepatic VLDL-apoB production and that additional metabolic processes responsible for diminished clearance of apoB-containing particles, associated with mutations in the lipoprotein lipase gene (9,10) and the apoAI/CIII/AIV gene cluster (43,44), are subordinate to this VLDL-apoB overproduction. Thus, metabolic processes associated with VLDL-apoB overproduction, such as increased delivery of free fatty acids to liver cells (45) or dysregulation of the action of microsomal triglyceride transfer protein required for assembly and secretion of hepatic VLDL-apoB (46), may have a genetic basis.

Recently, we demonstrated Mendelian inheritance of dense LDL subfraction profiles in these same FCH families (33). It appeared that the LDL subfraction profile was strongly related to the plasma apoB concentration, which raises the question whether the observed inheritance of the LDL subfractions more likely reflects the segregation of a trait in which affected subjects have elevated concentrations of apoB, as demonstrated in the present study. The few available reports, however, suggest that LDL subfraction profile phenotype and plasma apoB level genotype are separate Mendelian traits in FCH (2,47). Further analysis in which the LDL subfraction profile is considered independently of the genetic influence of apoB may elucidate this complicated issue.

In conclusion, there is a clear familial aggregation of elevated apoB concentrations that significantly correspond with the lipid phenotype in FCH families. Segregation analysis provides evidence that this aggregation is well explained by a single major gene effect with a codominant expression. Using these findings, linkage analysis can be used to explore the molecular defect involved in apoB regulation in FCH families.

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Chapter 9

Inherited susceptibility determines the distribution of dense low density lipoprotein subfraction profiles in familial combined hyperlipidemia

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ABSTRACT

Familial combined hyperlipidemia (FCH) is a heritable lipid disorder, in which dense low-density lipoprotein (LDL) subfraction profiles due to a predominance of small dense LDL particles are frequently observed. These small dense LDL particles are associated with cardiovascular disease. Using segregation analysis, we investigated to what extent these LDL subfraction profiles are genetically determined; also, the mode of inheritance was studied. Individual LDL subfraction profiles were determined by density gradient ultracentrifugation in 623 individuals of 40 well-defined Dutch FCH families. The individual LDL subfraction profile was defined as a quantitative trait by the continuous variable K, a reliable estimate of the relative contribution of each LDL subfraction to the overall profile. Variation in parameter K due to age, sex, and hormonal status was taken into account by introducing liability classes. Segregation analysis was performed by fitting a series of class D regressive models, implemented in the Statistical Analysis for Genetic Epidemiology (SAGE) program, after which genetic models were compared using log-likelihood ratio tests. Our data show that 60% of the variability of parameter K could be explained by lipid and lipoprotein levels and that a major autosomal locus, recessively inherited, with a population frequency of 0.42 ± 0.07, and an additional polygenic component of 0.21 best explained the clustering of atherogenic dense LDL subfraction profiles in these FCH families. Therefore, dense LDL subfraction profiles, associated with elevated lipid levels, appear to have a genetic basis in FCH.

INTRODUCTION

Familial combined hyperlipidemia (FCH) is the most common form of heritable lipid disorder and is accompanied by an increased incidence of cardiovascular disease (1). The trait is characterized by a multiple type hyperlipidemia in first-degree family members, with elevations of either total plasma cholesterol concentration, plasma triglyceride concentration, or both (2-4). Classically, FCH was thought to be inherited as a homogeneous single-gene disorder (1). Recent additional data, however, suggest that FCH is actually a more heterogeneous disorder. Overproduction of very-low- density lipoprotein (VLDL) apolipoprotein-B (apo-B) is supposed to be the primary cause (5). Delayed clearance of postprandial triglyceride-rich lipoproteins seems to be an aggravating metabolic defect in FCH (6). Mutations within the LpL gene, associated with a reduced hydrolytic capacity and elevated lipid levels (7,8), and in the apo-CI/AIV/CIII gene cluster (9) were genetically linked to the hyperlipidemia. Therefore, the expressed phenotypes may have a different etiology among families. The absence of a specific metabolic or genetic marker, combined with the characteristic variability of lipoprotein phenotypes among affected relatives complicates the diagnosis FCH in an individual patient, and still necessitates family investigation.

The increased risk of premature cardiovascular disease in FCH relatives is not completely explained by the sometimes mild elevation of VLDL and low density lipoprotein (LDL) alone. The predominance of small dense LDL particles, which are less rapidly processed by the LDL receptor (10) and more susceptible to oxidative modification (11-13), may also play a role. Although LDL heterogeneity is markedly influenced by age, gender, and environmental factors (14,15), an additional genetic influence controls the variability of LDL subfractions (14,16). Recently, evidence for linkage of the predominance of small dense LDL and a gene cluster near the LDL receptor locus on the short arm of chromosome 19 was reported (17) but has not yet been confirmed by others. Segregation analyses confirmed that the distribution of the qualitative LDL subfraction pattern in a FCH population has a genetic basis (18). LDL subfraction patterns could be classified as pattern A, consisting of large buoyant LDL particles, and pattern B, consisting of small dense LDL subfractions. Pattern B appeared to be inherited as a single-gene trait with a dominant or additive mode of inheritance in the FCH population. When LDL subfractions were separated by density gradient ultracentrifugation, a dichotomous classification of the LDL subfraction profile into pattern A and B could not fully reflect the great inter-individual variation in the LDL subfraction profiles (14). To describe the specific individual LDL subfraction distribution more accurately, we

introduced a continuous variable, parameter K, characterized by the relative contribution of the three major LDL subfractions LDL1, LDL2, and LDL3 to total LDL (14). Performing segregation analysis by using this parameter K, both inherited susceptibility and "environmental" factors (i.e., age, gender, body mass index [BMI], smoking habits, hormonal status in women, and lipid and lipoprotein levels) determined the LDL subfraction profile in 19 healthy Dutch families (14). In addition, longitudinal studies showed a remarkable consistency in time in the composition of the LDL subfraction profile both in untreated participants of the Framingham offspring study (19) and in FCH patients after drug treatment that substantially lowered particularly the plasma triglyceride concentrations (20,21). This predominance of subfractions containing small dense LDL particles irrespective of adequate lipid-lowering suggests that genetic factors may control LDL subfraction profiles in FCH subjects. It may therefore be questioned whether the LDL subfraction profile is a better metabolic marker for identifying affected relatives of FCH kindreds than are plasma lipids and lipoproteins.

Our study aimed to examine the inheritance of small dense LDL subfraction profiles in FCH on the basis of LDL particle density, as described by the quantitative parameter K. For this, a segregation analysis in a large sample of well-defined Dutch FCH families was performed using the Statistical Analysis for Genetic Epidemiology (SAGE) program. This approach may confirm that the LDL subfraction profile can be used as an additional diagnostic marker, which best reflects the metabolic and genetic influences of the lipid disorder in individual affected relatives of FCH families.

SUBJECTS and METHODS

Families

The recruitment of FCH kindreds took place via known affected probands who were attending the out-patient clinic of the University Clinics of Nijmegen (n=36) and Amsterdam (n=4). These probands exhibited a combined hyperlipidemia with both plasma cholesterol and triglyceride concentrations >90th percentile for age and gender during several periods in which they were not treated with lipid-lowering drugs, and despite dietary advice. In total, 40 multi-generational FCH kindreds containing 194 nuclear families with 687 individuals were used in this study. Families were included when a multiple type hyperlipidemia with elevated levels of total plasma cholesterol and/or triglycerides using the age- and sex-related 90th percentile upper levels of the prospective cardiovascular Münster (PROCAM) study (22) was present in first-degree relatives. Thus, besides the proband presenting a combined hyperlipidemia, the presence of one first-

degree relative with hypertriglyceridemia, hypercholesterolemia, or a combined hyperlipidemia was obligatory.

Families were excluded when first-degree family members had tendon xanthomata. None of the probands was homozygous for the apo E2 allele, and for all probands, a secondary cause (i.e., diabetes mellitus, hypothyroidism, and hepatic or renal impairment) for the presence of the hyperlipidemia could be excluded by standard laboratory tests. In addition, to refine the selection procedure, the 95th percentile for total plasma cholesterol and triglycerides was used if the BMI exceeded 30 kg/m^2 or an alcohol intake of more than two daily consumptions was present. All individuals were Caucasian above the age of 12 years. They all filled out a questionnaire about their medical status (i.e., diabetes, renal impairment and liver disease), medication use, alcohol intake, smoking habits, and hormonal status in women (pre- or postmenopausal, postmenopausal hormonal supplements, and oral contraceptive use). The study protocol was approved by the ethical committee of our institute.

Plasma

After an overnight fast and after withdrawal of lipid-lowering medication for at least 4 weeks, venous blood was drawn by venipuncture into K_3EDTA containing Vacutainer tubes. Non-local participants were visited at their homes, and blood was transported directly to the laboratory. Plasma was isolated within 3 h for determination of the lipid and lipoprotein levels, and the LDL subfraction profile.

Plasma lipid and lipoprotein assays

VLDL was isolated from whole plasma by ultracentrifugation at density = 1.006 g/ml for 16 h at 36,000 rpm in a fixed angle rotor (TFT 45.6 rotor, Kontron), in a Beckman L7-55 ultracentrifuge. High-density lipoprotein (HDL) cholesterol was determined by the polyethylene glycol 6000 method (23). LDL cholesterol was calculated by subtraction of VLDL cholesterol and HDL cholesterol from total plasma cholesterol. Total plasma cholesterol and triglyceride concentrations were determined by enzymatic, commercially available reagents (Boehringer-Mannheim, Germany, catalog. no. 237574 and Sera Pak, Miles, Belgium, catalog. no. 6639, respectively). Total plasma apo-B concentrations were determined by immunonephelometry (24). To achieve accurate results in relation to the Center for Disease Control Standardization Program, the obtained values were recalculated on the basis of an exchange of sera with Dr. S. Marcovina (Northwest Lipid Research Laboratory, Seattle WA, USA).

Low density lipoprotein subfractionation

LDL subfractions were detected by single spin density gradient ultracentrifugation, according to a method described elsewhere (25). After ultracentrifugation the LDL subfractions were visible as distinct bands in the middle of the tube. Up to five LDL subfractions could be distinguished concentrated in the following density ranges: LDL1 (1.030-1.033g/ml), LDL2 (1.033-1.040 g/ml), LDL3 (1.040-1.045 g/ml), LDL4 (1.045-1.049 g/ml), and LDL5 (1.049-1.054 g/ml). The ultracentrifugation tubes, containing the LDL subfractions stained with Coomassie Brilliant Blue R, were placed in a specially designed rack and photographed. Accurate documentation of the different LDL subfraction patterns was obtained by scanning the obtained slides in triplicate on a LKB 2202 ultrascan laser densitometer (Pharmacia LKB). The mean peak heights (h1-h5) of the LDL subfractions (LDL1-LDL5) on the three scans were used to calculate the variable K as a continuous variable, that best describes each individual LDL subfraction pattern (14). The relative contribution of each LDL subfraction, expressed by its peak height (%h1-%h5) relative to the total LDL subfraction profile, (total LDL [100%] = %h1+%h2+%h3+%h4+%h5), was calculated. The relative peak heights of LDL3 and the less frequently occurring LDL4 and/or LDL5 were added to give %h3'=(%h3+%h4+%h5), where LDL[100%] = LDL1[%h1] + LDL2[%h2] + LDL3[%h3']. When a subfraction profile was characterized by a predominance of buoyant LDL particles (h1-h3>0), variable K was calculated by K=(%h1-%h3')/(%h2-%h3'+1). In case of a predominance of heavy, dense LDL subfractions (h1-h3<0), variable K was calculated by: K=(%h1-%h3')/(%h2-%h1+1). A negative value (-1<K<0) reflects a dense subfraction profile, an intermediate subfraction profile is described by K=0, and a complete buoyant profile by a positive K value ($0 \leq K < 1$).

Statistical methods

To determine the difference in lipid and lipoprotein values in individuals with the buoyant ($K \geq 0$) and dense ($K < 0$) LDL subfraction profiles, these values were corrected for the influence of gender, age, BMI, smoking, and OC use. Adjusted lipid and lipoprotein values were standardized for 50-year-old, nonsmoking men with a BMI of 24 kg/m^2, and the difference between a buoyant and dense LDL subfraction profile in lipid and lipoprotein values was tested by using analysis of covariance. Pearson correlation coefficients were computed to determine correlations between parameter K and the variables age, BMI, and smoking. Partial correlation coefficients, controlling for the effect of age, BMI, smoking, and OC use, were computed to determine correlations between

parameter K and the variables total plasma cholesterol, plasma triglycerides, VLDL cholesterol and VLDL triglycerides, LDL cholesterol, HDL cholesterol, and apo-B.

Multiple linear-regression analysis was performed to determine the influence of gender, age, BMI, smoking, and OC use (independent variables) on parameter K (dependent variable). Stepwise multiple linear-regression was used to examine significant contributions of the independent variables (total plasma cholesterol, plasma triglycerides, VLDL cholesterol and VLDL triglycerides, LDL cholesterol, HDL cholesterol, and apo-B) to the prediction of the parameter K. Any influence of gender, age, smoking habits, BMI, and OC use was taken into account by forcing them in the models. Statistical analysis were performed by using the Statistical Analysis System computer programs (SAS Institute).

Segregation analysis

The segregation of the LDL subfraction profiles in FCH families was investigated by fitting a series of class D regressive models as implemented in SAGE release 2.2 (26), as described elsewhere (27). These models assume that variation among individuals for a quantitative trait is the result of a major gene effect and residual variation which may reflect both familial correlations and individual variations. Hypotheses were tested by fitting a general model and comparing its likelihood to that of reduced models representing specific modes of inheritance.

The general model used allows for two alleles at a single locus (denoted "L" for low and "H" for high) resulting in three types of individuals (LL, HL, and HH). The mean value of parameter K associated with each type is denoted μ(LL), μ(HL) and μ(HH). The within-type variance, σ^2, was assumed to be equal among all three types. The frequency of allele L is denoted q(L). The distribution of types in the population is assumed to be a Hardy-Weinberg equilibrium. Individuals of the LL, HL, and HH type are assumed to transmit the L allele with probabilities τ(LL), τ(HL), and τ(HH), respectively. In addition, the parameters ρ(SP), ρ(PO), and ρ(SS) denote the spouse, parent-offspring and sib correlations of parameter K, respectively. The class D models assume that correlations among all siblings are equal, but not necessarily because of common parentage alone. In case of equal parent-offspring and sibling correlations the class D models used have been shown to be mathematically and numerically equivalent to the conventional "mixed model" of inheritance in nuclear families (28). In this setting, the parent-offspring correlation provides an estimate of the polygenic heritability, i.e., $h^2 = 2\rho(PO)*\sigma^2/\sigma_T^2$, where σ_T^2 is the total variance and σ^2 is the variance conditional on the restricted model.

The general model was fitted and compared to various submodels by fixing certain parameters to specific values. For example, under a Mendelian model, transmission parameters are restricted to: $\tau(LL)=1$, $\tau(HL)=0.5$ and $\tau(HH)=0$, reflecting Mendelian segregation at a single locus with two alleles. A dominant model restricts $\mu HL=\mu LL$, while a recessive model restricts $\mu HL=\mu HH$. A restricted model is compared to the general model using the likelihood ratio test. When models are not hierarchical, the Akaike's information criterion (AIC) can be used to compare the fit of different models. For a given model, AIC=-2ln(L)+2*p (p=number of parameters estimated), and the model with the lowest AIC is considered to be the most parsimonious model (29).

To increase the power of the segregation analysis, the variation in the LDL subfraction profile (i.e., parameter K) was first standardized (mean=0, SD=1) within each of five liability classes (for men <50 years or ≥ 50 years, and for women pre- or postmenopausal, and for women using hormones), using the mean values obtained from spouses (n=93), to control for parameter K variation due to age, gender, and hormonal status. Initially, the segregation analysis was performed using these standardized values of K. Subsequently, also the unadjusted or crude K value with extra parameters for age, gender, pre- and post menopausal status were used in the segregation analysis as a check for the adjustment procedure.

In this study a correction for ascertainment bias was made because of a positive family-selection via known affected probands. The correction was made by conditioning the phenotypes of family members on those of the affected proband plus one extra affected (based on lipid levels) individual per kindred.

RESULTS

Study population

In total, 566 family members and 121 spouses in 40 FCH kindreds including 2 to 4 generations participated. Thirteen subjects of a non-informative branch of family #201 were excluded. Because of lack of the required volume of plasma (n=23) and technical errors (n=28), the LDL subfraction profiles of 51 subjects (23 FCH relatives and 28 spouses) could not be determined. However, plasma lipid and lipoprotein levels of these 51 subjects were taken into account in order to support the diagnosis FCH. In total, 623 subjects (299 men and 324 women) were included in the segregation analysis of parameter K.

Analysis of LDL subfraction profiles

In all sera, Three to five distinct LDL subfraction bands, contributing to the individual LDL subfraction profiles could be distinguished. The frequency distribution of the various LDL subfraction profiles in spouses and in FCH relatives, described by parameter K and standardized within each of 5 liability classes, is presented in figure 1.

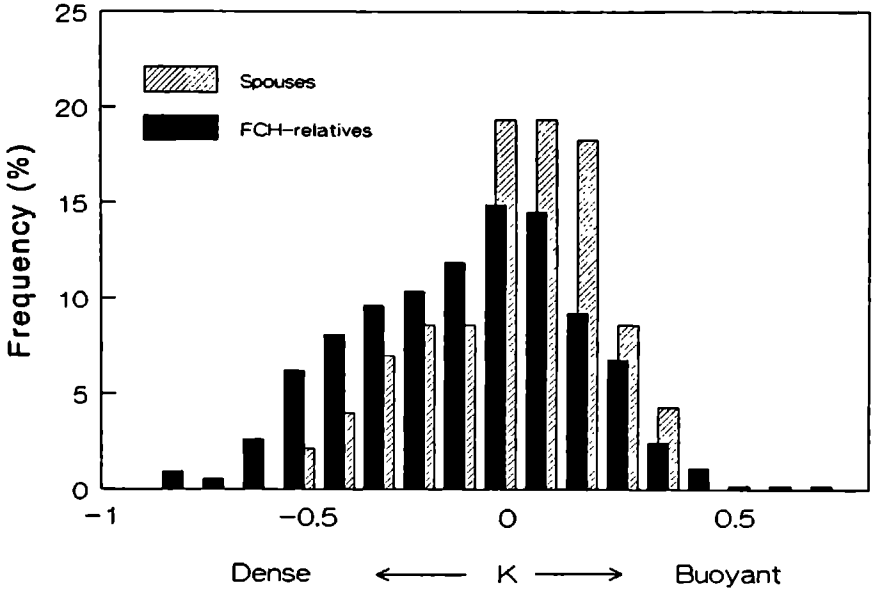

Figure 1 Frequency distribution of values for the standardized parameter K among FCH relatives (n=530) and spouses (n=93). A buoyant LDL subfraction profile is defined by $K \geq 0$, and a dense LDL subfraction profile by $K < 0$.

FCH relatives showed a skewed distribution with a predominance of dense (i.e., more negative values of parameter K) LDL subfraction profiles, while in spouses more buoyant profiles were observed. The mean value of parameter K was different in each liability class, underlining a relation with age, gender, and hormonal status in women (table 1). In all 5 liability classes the value of K was lower, or tended to be, in the FCH relatives than in the spouses, reflecting the predominance of dense LDL subfraction profiles in FCH subjects independent of age, gender, and female hormonal status (table 1). Further, the mean value of parameter K is lower among men and decreased with age in both sexes (table 1).

Table 1: Distribution of LDL subfraction profiles defined by parameter K, in 5 liability classes on the basis of gender, age and hormonal status

		Liability Class				
		Male		Female		
		<50 years	≥50 years	premenopausal not using OC	taking hormones[a]	postmenopausal
No. of subjects	Spouses	59[b]	25	16	7	29
	FCH	173	85	104	75	93
Age (years)	Spouses	30.7 ± 7.3	59.4 ± 6.0	44.4 ± 8.1	37.4 ± 12.6	57.7 ± 7.7
	FCH	31.2 ± 10.5	60.5 ± 7.0	33.0 ± 12.3	29.5 ± 8.6	60.5 ± 8.0
BMI (kg/m²)	Spouses	23.0 ± 2.0	26.5 ± 3.1	24.9 ± 3.6	21.4 ± 2.1	26.8 ± 3.5
	FCH	24.0 ± 3.8	26.1 ± 2.9	23.9 ± 3.9	23.2 ± 3.2	26.3 ± 3.9
K value	Spouses	-0.07 ± 0.26	-0.22 ± 0.23	0.07 ± 0.20	-0.10 ± 0.15	-0.19 ± 0.24
	FCH	-0.27 ± 0.29**	-0.38 ± 0.29*	-0.09 ± 0.31	-0.14 ± 0.24	-0.30 ± 0.32

Values are presented as mean ± SD; FCH means total population of FCH relatives; [b]data for spouses in this liability class are obtained from a previous study by de Graaf et al. (ref. no. 17); OC, oral contraceptives; [a]this liability class contains both premenopausal women using OC (spouses n=5, FCH n=72) and postmenopausal women using hormonal replacement (spouses n=2, FCH n=3); *p<0.01, **p=0.001.

Women using OC and postmenopausal women using hormonal replacement (both presented in one liability class) had a lower mean value of K than premenopausal women not using OC (table 1).

Interrelation of the LDL subfraction profile with anthropometric measurements

The mean age, BMI, and percentage of smokers among all FCH relatives (men and women) are presented in table 2. The values of parameter K correlated significantly with age and BMI and weakly with smoking habits. These data indicate that with increasing age and BMI and with smoking, a denser LDL subfraction profile was observed (table 2). Multiple regression analysis of parameter K, including age, BMI, smoking habits, gender and OC use, revealed that these variables together contributed 24% to the variation of parameter K. The prevalence of more dense LDL subfraction profiles in the FCH population is higher (figure 1), and in accordance with the findings in the spouses, the prevalence in this FCH population also increases with age, BMI, the gender and OC use (table 1).

Interrelation of the LDL subfraction profile with lipid and lipoprotein levels

The lipid and lipoprotein levels of all FCH relatives, adjusted for age, gender, BMI, and smoking habits, and stratified by buoyant and dense LDL subfraction profiles are presented in table 2. Within FCH relatives, parameter K was found to be negatively correlated with apo-B, plasma triglycerides, VLDL triglycerides, VLDL cholesterol and total plasma cholesterol and positively with HDL-cholesterol concentrations. Therefore, with decreasing parameter K values, a lipoprotein profile is observed, characterized by higher concentrations of total apo-B, plasma triglyceride, and total plasma cholesterol, and by a lower HDL cholesterol concentration. These associations are also observed if buoyant LDL subfraction profiles are compared with dense LDL subfraction profiles (table 2). The LDL cholesterol concentration itself did not differ between buoyant and dense LDL subfraction groups.

Because we anticipated strong intercorrelations between lipid and lipoprotein levels, stepwise multiple regression analysis was performed to evaluate which combination of lipids and lipoproteins could be used to predict the LDL subfraction profile both in spouses and in FCH relatives (table 3). In order to be able to compare our results with those from the literature, where data from VLDL cholesterol, VLDL triglycerides and,LDL cholesterol are frequently unavailable, the multiple regression analysis was performed twice, once excluding VLDL and LDL concentrations (model I) and once

including values of VLDL and LDL (model II).

Table 2: Adjusted lipids and (apo)lipoproteins for FCH relatives stratified by buoyant ($K \geq 0$) and dense ($K < 0$) LDL subfraction profiles.

	All FCH relatives (n=530)	Buoyant ($K \geq 0$) (n=139; 26.2%)	Dense ($K < 0$) (n=391; 73.8%)	Correlation coefficient (all)	p value
Age (years)	41.1 ± 16.9	36.4 ± 16.6	42.7 ± 16.6	-0.31	0.0001
BMI (kg/m^2)	24.5 ± 3.7	22.6 ± 3.0	25.3 ± 3.8	-0.42	0.0001
Smoking ($\geq$ 1/day)	27.2%	16.3%	31.9%	-0.10	0.02
Total cholesterol[a]	6.03 ± 0.09	5.68 ± 0.13	6.12 ± 0.13	-0.22	0.0001
Triglycerides[a]	2.82 ± 0.02	0.97 ± 0.06	3.69 ± 0.04	-0.42	0.0001
VLDL-cholesterol[a]	0.98 ± 0.06	0.62 ± 0.10	1.05 ± 0.06	-0 41	0.0001
VLDL-triglycerides[a]	0.91 ± 0.06	0.22 ± 0.08	1.26 ± 0.05	-0.44	0.0001
LDL-cholesterol[a]	3.98 ± 0.08	3.80 ± 0.12	4.03 ± 0.08	-0.06	NS
HDL-cholesterol[a]	1.10 ± 0.02	1.29 ± 0.03	1.06 ± 0.02	0.50	0.0001
Apo-B[b]	140.2 ± 2.6	118.6 ± 3.7	145.3 ± 2.6	-0.50	0.0001

Values are presented as mean ± SE. Values are adjusted to those of 50-year-old, non-smoking men with a BMI of 24 kg/m^2; [a]concentrations in mmol/l; [b]concentration in mg/dl; NS=not significant

Both models included age, gender, BMI, smoking habits, and OC use as covariates. In spouses, a significant contribution of plasma triglycerides, HDL cholesterol, and apo-B to the prediction of parameter K (R^2=0.64) was found. VLDL cholesterol, VLDL triglycerides, and LDL cholesterol (entered into model II) did not contribute to the prediction of parameter K in spouses (table 3). In FCH relatives, a significant contribution of plasma triglycerides, HDL cholesterol and apo-B to the prediction of K was found as well (model I). However, these parameters accounted for 58% of the variability of parameter K. When VLDL triglycerides, VLDL cholesterol, and LDL cholesterol were included in the analysis (model II), VLDL triglycerides and VLDL cholesterol significantly contributed to predicting parameter K of FCH relatives, but LDL cholesterol did not. So, inclusion of VLDL in the model provided little additional information in the prediction of the LDL subfraction profile of FCH relatives.

Table 3: Regression coefficients and levels of significance for variables in models most appropriate for predicting the LDL subfraction profile, defined by parameter K, of spouses and FCH relatives

	Model II		Model I		Model II	
Variable	Spouses	p-value	FCH relatives	p-value	FCH relatives	p-value
Intercept	-0.336 ± 0.208	0.110	-0.221 ± 0.096	0.021	-0.359 ± 0.100	0.0003
Sex: Group 1[a,b]	-0.021 ± 0.039	0.584	-0.048 ± 0.021	0.021	-0.033 ± 0.021	0.125
Sex: Group 2[a,c]	-0.116 ± 0.078	0.144	-0.044 ± 0.029	0.130	-0.039 ± 0.029	0.189
Age (years)[a]	-0.001 ± 0.002	0.534	0.0002 ± 0.0007	0.817	0.0001 ± 0.0001	0.919
Body mass index(kg/m^2)[a]	0.008 ± 0.006	0.131	-0.002 ± 0.003	0.509	-0.0001 ± 0.003	0.972
Smoking[a]	0.086 ± 0.041	0.038	0.024 ± 0.020	0.246	0.036 ± 0.020	0.076
Total cholesterol[d]	-[e]	-	-[e]	-	-[e]	-
Log Triglycerides[d]	-0.178 ± 0.054	0.002	-0.219 ± 0.024	0.0001	-0.106 ± 0.061	0.082
VLDL-cholesterol[d]	-[e]	-	-[f]	-	0.075 ± 0.019	0.0001
Log VLDL-triglycerides[d]	-[e]	-	-[f]	-	-0.146 ± 0.041	0.0004
LDL-cholesterol[d]	-[e]	-	-[f]	-	-[e]	-
HDL-cholesterol[d]	0.268 ± 0.062	0.0001	0.214 ± 0.034	0.0001	0.201 ± 0.034	0.0001
Apo-B[g]	-0.0002 ± 0.0001	0.008	-0.0001 ± 0.0001	0.014	-0.0001 ± 0.0001	0.002
R^2	0.64		0.58		0.60	

[a]Variables forced into models; [b]men versus women not taking oral contraceptives (OC); [c]women taking OC versus women not taking OC; [d]this variable does not contribute significantly; [f]This variable is not included in this model; [g]in mg/dl

Segregation analysis

Segregation analysis was carried out using the standardized parameter K (table 4). Analysis using the unadjusted parameter K yielded the same results (data not shown). Table IV presents parameter estimates for 11 different modes of inheritance.

In a sporadic or unimodal model, the spouse, parent-offspring, and sibling correlations were examined to determine multifactorial or polygenic influences. The spouse correlation (ρ(SP)) was found to be negligible (0.04 ± 0.11) and was therefore fixed at 0 in all models. The parent-offspring correlation (ρ(PO)) and sibling correlation were found to be equal (0.22 ± 0.04). Therefore, in order to estimate a possible polygenic component, all subsequent models assumed a single correlation among first-degree relatives ($\rho(SS)=\rho(PO)$). The sporadic models (model 1 and 2) fitted the data significantly worse than the unrestricted general model. This general model (model 11), with all variables unrestricted, revealed strong evidence for inheritance of parameter K, with transmission parameters $\tau(LL)=0.93$, $\tau(HL)=0.52$ and $\tau(HL)=0.10$, almost equal to values expected under Mendelian transmission ($\tau(LL)=1$, $\tau(HL)=0.5$ and $\tau(HL)=0$). Therefore at least a bimodal distribution of parameter K was inferred. To consider non-genetic transmission, models 9 and 10, in which transmission parameters were set equal to q(L), were examined. These models fit poorly and could be rejected. In order to examine the mode of inheritance, dominant models (models 3 and 4), recessive models (models 5 and 6), and codominant models (models 7 and 8) were fitted by fixing the transmission parameters at their Mendelian expectations (i.e., $\tau(LL)=1$, $\tau(HL)=0.5$ and $\tau(HL)=0$). Dominant models of inheritance could be rejected against the general model ($p<0.01$). The fit of the recessive model of inheritance was found to be equal to the general model. This was true, however, only if the residual correlation was considered (model 6). This residual correlation is equivalent to the polygenic component within the nuclear familiy, and represents $2\rho(PO)^*\sigma^2/\sigma_T^2$ (=21%) of the variation in adjusted LDL subfraction profiles. A codominant major gene model of inheritance (trimodal distribution of values for parameter K) as well as a single recessive model (models 5 and 7) fit the data worse than the general model. However, when residual correlations were considered neither the codominant nor the recessive model could be rejected (i.e., models 6 and 8). Model 6 has the lowest AIC value and appears to be the most parsimonious model, although model 8 is a close second.

Table 5 presents the predicted K values under model 6 (recessive mode) and model 8 (codominant mode). The predicted values for the LH type are very similar to those for the HH type, indicating that the most parsimonious model is the recessive one.

Table 4 Segregation analysis of adjusted subfraction profiles in 623 members of 40 FCH kindreds

Model	q(L)	μ(LL)	μ(LH)	μ(HH)	σ^2	τ(LL)	τ(LH)	τ(HH)	ρ(PO)	ρ(SS)	-2ln(L)	AIC	X^2
1 Sporadic	[1 0]	-0 47			1 74				[0 00]	[0 00]	1845 6	1847 6	89 9***
		±0 06			±0 11								(8 df)
2 Sporadic+ρ(PO/SS)	[1 0]	-0 28			1 71				0 23	=ρ(PO)	1787 5	1793.5	31 8***
		±0 09			±0 11				±0 04				(7 df)
3 L Dominant	0 12	-1 58	=μ(LL)	0 25	0 94	[1 0]	[0 5]	[0 0]	[0 00]	[0 00]	1779 8	1787 8	24 1***
	±0 03	±0 12		±0 09	±0 09								(6 df)
4 L Dominant+ρ(PO/SS)	0 11	-1 61	=μ(LL)	0 23	0 97	[1 0]	[0 5]	[0 0]	0 13	=ρ(PO)	1771 8	1781 8	16 1**
	±0 03	±0 15		±0 10	±0 11				±0 05				(5 df)
5 L Recessive	0 47	1 69	=μ(HH)	0 24	0 87	[1 0]	[0 5]	[0 0]	[0 00]	[0 00]	1773 1	1781 1	17 4**
	±0 04	±0 13		±0 07	±0 09								(6 df)
6 L Recessive+ρ(PO/SS)	0 42	1 92	=μ(HH)	0 17	0 90	[1 0]	[0 5]	[0 0]	0 20	=ρ(PO)	1762 0	1772 0	6 3
	±0 07	±0 28		±0 12	±0 13				±0 11				(5 df)
7 Codominant	0 43	-1 84	0 02	0 64	0 79	[1 0]	[0 5]	[0 0]	[0 00]	[0 00]	1767 5	1777 5	11 8*
	±0 04	±0 14	±0 11	±0 15	±0 09								(5 df)
8 Codominant+ρ(PO/SS)	0 42	-1 90	0 09	0 46	0 83	[1 0]	[0 5]	[0 0]	0 14	=ρ(PO)	1760 9	1772 9	5 2
	±0 04	±0 15	±0 12	±0 23	±0 10				±0 07				(4 df)
9 Environmental	0 53	-2 02	-0 07	0 59	0 74	=q(L)	=q(L)	=q(L)	[0 00]	[0 00]	1820 9	1830 9	65 2***
	±0 04	±0 14	±0 18	±0 28	±0 13								(5 df)
10 Environmental+ρ(PO/SS)	0 44	1 76	-0 01	-0 01	1 22	=q(L)	=q(L)	=q(L)	0 28	=ρ(PO)	1782 7	1794 7	27 0***
	±0 09	±0 28	±0 55	±0 84	±0 20				±0 05				(4 df)
11 General	0 40	1 95	-0 07	0 58	0 76	0 93	0 53	0 13	0 06	0 17	1755 7	1775 7	Reference
	±0 06	±0 15	±0 16	±0 20	±0 10	±0 06	±0 08	±0 09	±0 10	±0 09			

Values are presented as mean ± SE, *p<0 05, **p<0 01, ***p<0 001, [ ] = restricted

Table 5 Unadjusted (i.e., crude) K values of models 6 and 8 in table 4 for the five liability classes

Genotype:	LL	LH	HH
Model 6 (Recessive):			
Men <50 years	-0.59 ± 0.07	=HH	-0.04 ± 0.03
Men ≥50 years	-0.69 ± 0.07	=HH	-0.19 ± 0.02
Women premenopausal, not taking OC	-0.33 ± 0.06	=HH	0.10 ± 0.02
Women taking hormones[a]	-0.40 ± 0.04	=HH	-0.08 ± 0.01
Women postmenopausal	-0.67 ± 0.07	=HH	-0.15 ± 0.02
Model 8 (Codominant):			
Men <50 yrs	-0.57 ± 0.04	-0.05 ± 0.03	0.04 ± 0.06
Men ≥50 yrs	-0.67 ± 0.03	-0.21 ± 0.03	-0.12 ± 0.05
Women premenopausal, not taking OC	-0.31 ± 0.03	0.09 ± 0.02	0.17 ± 0.05
Women taking hormones[a]	-0.38 ± 0.02	-0.09 ± 0.02	-0.03 ± 0.03
Women postmenopausal	-0.64 ± 0.04	-0.19 ± 0.03	-0.08 ± 0.05

Values are presented as mean ± SE; L and H are two alleles at a single locus; allele L is associated with the deviant phenotype; allele H is associated with a normal phenotype; [a]including premenopausal women using OC (n=77) and postmenopausal women taking hormonal replacement (n=5)

DISCUSSION

To examine a possible genetic basis for the LDL subfraction profile distribution in patients with FCH, we investigated the segregation of individual LDL subfraction profiles (parameter K), in a large sample of well-defined Dutch FCH families. The results of our study show that the observed clustering of the dense, probably atherogenic, LDL subfraction profiles in this FCH population indeed has a genetic basis, best described by a common, major autosomal gene with a population frequency of 0.42 ± 0.07 and a recessive mode of inheritance with a polygenic heritability component of 0.25.

Several studies have suggested possible genetic control of LDL heterogeneity, using a variety of techniques to detect LDL heterogeneity and to analyze its genetic susceptibility (14,30,31). We performed a segregation analysis in a FCH population, using LDL subfraction profiles obtained by density gradient ultracentrifugation, and described by a continuous parameter K. Most reports on LDL subfractions, including the only paper

published thus far about the inheritance of LDL subfractions in FCH, distinguish only 2 distinct LDL subfraction patterns, denoted "A" (parameter $K \geq 0$) and "B" (parameter $K < 0$), on the basis of LDL particle size of the major LDL subfraction, obtained by gradient gel electrophoresis (18). Additional analysis by Austin et al. using the LDL peak particle diameter as continuous trait was not superior to that of the dichotomous classification in determining the mode of inheritance of LDL subfraction patterns in primarily healthy families, probably because it was based only on the major LDL subfraction of a profile (32). The quantitative parameter K, which allows the contribution of all LDL subfractions to be taken into account, reflects better the great inter-individual variation in the LDL subfraction profile (14). When a quantitative trait such as the LDL subfraction profile is reduced to a dichotomy, i.e., LDL subfraction profile "A" and "B", much information is lost, since it is unknown whether an individual is close or far from the threshold. In addition, the general or mixed model was originally formulated in terms of quantitative traits. Indeed, we have shown in a normolipidemic population that segregation analyses on the basis of the quantitative parameter K discriminated better than on the basis of a dichotomous A or B classification, with regard to models of inheritance of LDL subfraction profiles (14).

The only study to which we could compare our data is a segregation analysis of LDL subfraction patterns in seven FCH kindreds using the binary trait classification (18). They found that the heavy pattern "B" was inherited as an autosomal trait with either a dominant or an additive mode of inheritance, and a small multifactorial inheritance component. The population frequency for the proposed pattern "B" allele in FCH was 0.30, which was very similar to the frequency in a healthy Mormon population (0.25) (33). Our results more strongly suggest inheritance of LDL subfraction profiles through a single major locus. However, in contrast to Austin et al. our data indicate a recessive mode of inheritance with a polygenic component with a gene frequency of 0.42. This gene frequency is higher than the 0.19 found in a healthy Dutch population with a recessive mode of inheritance (14).

In assessing the validity of our conclusions from this study, several factors must be considered. First, we transformed the parameter K in order to increase the power of the segregation analysis. The classification by introducing five liability classes can be criticized. The analysis with an unadjusted K-value, however, revealed similar results. Inadequate correction for ascertainment bias can also provide misleading results, suggesting the presence of a major locus when one is absent (34). The way in which adequate correction for ascertainment bias should be carried out is still under debate. We

conditioned the phenotypes of family members on two affected individuals per family.

It appeared that 60% of the variation in parameter K could be explained by gender, age, BMI, smoking habits, hormonal status in women, and lipid and lipoprotein levels. This rate confirms the results of previous reports, where ≈60% of the variability of the LDL subfraction profile could be explained by anthropometric measurements and metabolic influences (14,33,35). Therefore, the findings of a strong interaction between plasma lipids and lipoproteins (i.e., plasma triglycerides, apo-B, and LDL subfraction profiles), is very consistent. This raises the question whether the observed inheritance of LDL subfractions is more likely the segregation of a trait in which affected subjects have elevated concentrations of plasma triglycerides and apo-B, a diagnostic feature in FCH families. This hypothesis, however, is not supported by recent reports. Cullen et al. provide evidence for a major gene acting on serum triglyceride levels in 55 British FCH families (36). From their data, they also presumed the presence of exacerbating genes, such as a major gene acting on LDL subfraction profiles. This gene would have an allele that confers high triglyceride levels on those subjects with FCH. Jarvik et al. observed Mendelian segregation of a major locus for apo-B in seven FCH families, which was not eliminated by adjustment for dichotomously classified LDL subfraction pattern (37). They concluded that LDL subfraction phenotype and apo-B level genotype are separate Mendelian traits in FCH. Hokanson et al. recently reported that the physical and chemical properties of LDL particles in FCH differ from those of normolipidemic controls. Many of these properties appear to be independent of plasma triglyceride concentrations and are only found in FCH patients (38). In addition, we have demonstrated Mendelian transmission of LDL subfraction profiles in healthy normolipidemic Dutch families, using the same LDL subfraction isolation technique. In this normolipidemic population, a significant intercorrelation between lipids, lipoproteins, and LDL subfraction profiles was observed, comparable to that in the present FCH population. So, the distribution of LDL subfraction profiles in healthy Dutch normolipidemic families is the result of a combination of underlying genetic traits and environmental or behavioral traits. Our analysis indicates that the appearance of a dense LDL subfraction profile is not unique for affected FCH patients, but in the latter group it is more frequently observed.

There are several arguments, obtained from this study and previous work, for considering small dense LDL as an additional metabolic marker for FCH. First, we observed the same mode of inheritance of a major locus for the LDL subfraction profile in a healthy population (14) and a FCH population (present study). The twice-as- high gene frequency of the proposed allele for the trait suggests a higher frequency of the allele for a

dense LDL subfraction profile in FCH. Also, the fact that the LDL subfraction profile in FCH subjects is reasonably refractory to lipid-lowering therapy (20,21), suggests that it is an underlying metabolic marker. A third argument is the observed Mendelian transmission of dense LDL subfraction profiles in these FCH kindreds, although selection of the families took place on the basis of elevated lipid level. At the same time, we demonstrated that these lipid concentrations could only explain 60% of the variation in the profiles.

In conclusion, a constant individual LDL subfraction profile controlled by a major recessive locus is associated with the elevated and intra-individual fluctuating lipid levels in FCH. The predominance of dense LDL subfraction profiles appears to reflect both the metabolic and genetic influences on the lipid disorder. Therefore, additional determination of the LDL subfraction profile may be more informative in the search for affected FCH relatives than is lipid phenotyping alone.

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Chapter 10

Separate genetic mechanisms determine plasma apolipoprotein-B levels and dense LDL subfraction distribution in familial combined hyperlipidemia

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ABSTRACT

Familial combined hyperlipidemia (FCH) is a common heritable lipid disorder, characterized by elevations of plasma cholesterol and/or triglycerides within first-degree relatives and an elevation of plasma apolipoprotein-B (apoB). Furthermore, a predominance of small dense atherogenic low density lipoprotein (LDL) particles, consistently associated with the elevated plasma lipid levels, is frequently observed. Previously, we demonstrated a major recessive locus control of dense LDL profiles inheritance and a codominant mechanism involved in the aggregation of elevated plasma apoB levels in 623 individuals of 40 well-defined FCH families. Elevated plasma apoB is metabolically associated with dense LDL in FCH. To establish whether distinct genetic mechanisms express both traits, we now evaluated the inheritance of small dense LDL subfraction profiles after correction for the predicted genetic influence of a putative apoB locus. By means of a segregation analysis of apoB, individual apoB genotypes (i.e., AA, AB, or BB) could be assigned to 85% of all individuals. LDL subfraction profiles expressed by a continuous variable K differed significantly between apoB genotypes, supporting the metabolic relation between apoB and LDL subfraction profile. Subsequently, LDL subfraction profiles were adjusted for predicted apoB influence by subtracting the apoB genotype specific mean K value from the individual K values. These apoB genotype specific mean K values were obtained from those individuals with their apoB genotype probability $\geq 70\%$. Segregation analysis of residual LDL subfraction profiles provided substantial evidence for a major locus inheritance pattern. Therefore, it is concluded that two distinct genetic mechanisms influence the appearance of elevated plasma apoB and the predominance of dense LDL in these FCH families.

INTRODUCTION

Familial combined hyperlipidemia (FCH) is the most common form of heritable lipid disorders with an estimated population frequency of 1 to 2%. At least 10% of all premature cardiovascular disease can be ascribed to this lipid disorder (1,2). The hyperlipidemia is characterized by elevations of the total plasma cholesterol concentration and/or the plasma triglyceride concentration in first-degree relatives and is therefore also known as 'multiple type hyperlipidemia'. Other characteristics of the disorder are an elevated plasma apolipoprotein-B100 (apoB) concentration, a decreased high density lipoprotein (HDL) concentration, and a preponderance of small dense low density lipoprotein (LDL) particles (2-4).

Originally, FCH was thought to be inherited as a homogeneous single gene disorder with a major effect on triglyceride levels and a secondary effect on plasma cholesterol levels (1). Although recently evidence has been provided for such a major locus effect on triglyceride levels, it was considered that a number of different loci may affect the expression of the FCH phenotype and that the major genes involved still have to be identified (5). Other studies also have shown FCH to be a heterogeneous lipid disorder, comprising features of the insulin resistance syndrome, hyperapobetalipoproteinemia and familial dyslipidemic hypertension (3,6-8). Interestingly, all of these entities are associated with the presence of small dense LDL (9). Still, the absence of a specific metabolic or genetic marker complicates the diagnosis of those entities which are probably lumped together under the rubric of FCH.

FCH is considered to be caused by hepatic overproduction of very low density lipoprotein (VLDL)-apoB (10,11), resulting in elevated levels of apoB-containing lipoproteins in the density range of VLDL, intermediate density lipoprotein (IDL) and LDL particles. A delayed clearance of the triglyceride-rich apoB-containing lipoproteins, in part caused by impaired lipoprotein lipase activity may modify the lipid phenotypic expression (12,13). Using segregation analysis, the variation in age- and sex-adjusted apoB levels has been ascribed to a yet unmapped codominant mendelian locus (14,15). This locus was later found to be predictive of FCH (16,17). It is noteworthy that elevated plasma apoB levels, corresponding with elevated VLDL and/or LDL levels in FCH, have been metabolically associated with the predominance of small dense LDL (18-20). Both elevated plasma apoB and small dense LDL are associated with the occurrence of cardiovascular disease (21-24), although there may be some overlap in the reported respective cardiovascular risks related to each entity.

Also the distribution of small dense LDL subfraction profiles appears to have a

genetic basis in FCH (4,25). Because the mechanisms reported for the inheritance of plasma apoB levels were different from those reported for the inheritance of LDL subfraction profiles in FCH, one may wonder whether the presumed inheritance of LDL subfractions is actually caused by the segregation of a trait responsible for elevated plasma apoB concentrations. After all, with two traits involved in the same metabolic pathway, the major locus controlling one phenotype may have a carryover effect on the other phenotype. In order to investigate whether distinct genetic mechanisms underlie the familial aggregation of elevated plasma apoB levels and the predominance of dense LDL subfraction profiles observed in FCH, we conducted a segregation analysis of LDL subfraction profiles adjusted for the inferred genotypic influence of a putative apoB locus. Elucidation of the relation between these two traits and depiction of their genetic basis in FCH would be helpful in the search for molecular defects involved.

MATERIALS AND METHODS

Study Population

The FCH pedigrees considered here have been studied for inheritance of LDL subfraction profiles (25) and plasma apoB levels (26). The families were ascertained through probands exhibiting a combined hyperlipidemia with both plasma cholesterol and triglyceride concentrations above the 90th percentile, adjusted for age and gender, as obtained from the Prospective Cardiovascular Münster (PROCAM) Study (27). These values were consistent over several measurements in which the probands had not been given any lipid-lowering drugs. Families were included when a so called 'multiple type hyperlipidemia' (2) with levels of total plasma cholesterol and/or triglycerides above the 90th percentile was present. This means that besides the proband, at least one first-degree relative exhibited hypercholesterolemia or hypertriglyceridemia.

All participants completed a questionnaire on medical status, medication use, alcohol intake and smoking habits. All probands were tested for an underlying cause of the hyperlipidemia, i.e., diabetes mellitus, hypothyroidism and hepatic or renal impairment. The presence of one of these causes excluded them and their families from further analysis. None of the probands in these families was homozygous for the apo $\varepsilon 2$ allele and none of the first-degree relatives had tendon xanthomata. In addition, to refine the selection procedure the 95th percentiles for plasma cholesterol and triglycerides were used if the body mass index exceeded 30 kg/m^2, or an alcohol consumption of more than two drinks per day was present. All individuals were Caucasians above the age of 10 years. The study protocol was approved by the ethical committee of the University

Hospital Nijmegen.

Plasma

After an overnight fast and a withdrawal of lipid-lowering medication for at least four weeks, blood was drawn by venipuncture into EDTA-containing vacutainer tubes. Non-local participants were visited at their homes, and blood was transported directly to the laboratory. Plasma was isolated within 3 hours for determination of the lipid and lipoprotein levels and LDL subfraction profiles. Remaining plasma was stored at -80^{0}C for plasma apoB determination.

Measurement of Lipid, Lipoprotein and Apolipoprotein levels

VLDL was isolated from whole plasma by ultracentrifugation at density=1.006 g/ml for 16 hours at 36,000 rpm in a fixed angle rotor (TFT 45.6 rotor, Kontron, Zürich), in a Beckman L7-55 ultracentrifuge (Beckman, Palo Alto, CA, U.S.A.). High density lipoprotein (HDL) cholesterol was determined by the polyethylene glycol 6000 method (28). LDL cholesterol was calculated by subtraction of VLDL cholesterol and HDL cholesterol from total plasma cholesterol. Cholesterol and triglyceride concentrations were determined by enzymatic, commercially available reagents (Boehringer-Mannheim, Germany, cat. No.237574 and Sera Pak, Miles, Italy cat. No. 6639, respectively). Total plasma apoB concentrations were determined by immunonephelometry (29). To achieve accurate results in relation to the Center for Disease Control Standardization Program, obtained values were recalculated on the basis of an exchange of sera with Dr. S. Marcovina (Northwest Lipid Research Laboratory, Seattle, WA, U.S.A.).

Low density lipoprotein subfractionation

LDL subfractions were detected by single spin density gradient ultracentrifugation, according to a method described in detail elsewhere (30). Up to five LDL subfractions could be distinguished concentrated in the following density ranges: LDL1 (1.030-1.033g/ml), LDL2 (1.033-1.040 g/ml), LDL3 (1.040-1.045 g/ml), LDL4 (1.045-1.049 g/ml), and LDL5 (1.049-1.054 g/ml). The ultracentrifugation tubes, containing the LDL subfractions stained with Coomassie Brilliant Blue R, were placed in a specially designed rack and photographed. Accurate documentation of the different LDL subfraction patterns was obtained by scanning the obtained slides in triplicate on a LKB 2202 ultrascan laser densitometer (Pharmacia LKB). The mean peak heights (h1-h5) of the LDL subfractions (LDL1-LDL5) on the three scans were used to calculate the variable K as a continuous

variable, that best describes each individual LDL subfraction pattern (31,32). A negative value ($-1 < K < 0$) reflects a dense subfraction profile, whereas a complete buoyant profile reveals a positive K value ($0 \leq K < 1$) (31).

Statistical Analysis

To determine the difference in lipid and lipoprotein values in the FCH probands, the remaining FCH relatives and the spouses, these values were first adjusted for the influence of gender, age, BMI, and smoking by lineair regression. Differences in mean adjusted lipid and lipoprotein values were tested by analysis of variance. Because of a skewed distribution of both variables, a Spearman correlation coefficient was calculated to depict the relation between plasma apoB concentrations and crude K values.

Segregation analyses

The preceding segregation analyses of LDL subfraction profiles and standardized plasma apoB levels (25,26), as well as the present segregation analysis of LDL subfraction profiles adjusted for the inferred genotypic influence of a putative apoB locus were all performed by fitting a series of class D regressive models (33) implemented in the Statistical Analysis for Genetic Epidemiology (SAGE) release 2.2 software package (34). These models assume that variation among individuals in a quantitative trait is the result of a major gene effect and residual variation which may reflect both familial correlations and individual variations (35). Hypotheses were tested by fitting a general model and comparing its likelihood to that of reduced models representing specific modes of inheritance. The general model used allows for two alleles at a single locus (denoted "L" for low and "H" for high) resulting in three types of individuals (LL, HL, and HH). The mean value of adjusted parameter K associated with each type is denoted μ(LL), μ(HL) and μ(HH), and the mean value of standardized plasma apoB associated with each type is denoted μ(AA), μ(AB) and μ(BB) (26). The within-type variance, σ^2, was assumed to be equal among all three types. The frequency of allele L is denoted q(L). Individuals of the LL, HL, and HH type are assumed to transmit the L allele with probabilities τ(LL), τ(HL), and τ(HH), respectively. In addition, the parameters ρ(SP), ρ(PO), and ρ(SS) denote the spouse, parent-offspring and sib correlations of the adjusted parameter K, respectively. The class D models assume that correlations among all siblings are equal, but not necessarily because of common parentage alone. In case of equal parent-offspring and sibling correlations the class D models have been shown to be mathematically equivalent to the conventional "mixed model" of inheritance in nuclear families (36). In

this case, the parent-offspring correlation provides an estimate of the polygenic heritability, i.e., $h^2 = [2\rho(PO)]*[\sigma^2/\sigma_T^2]$, where σ_T^2 is the total variance and σ^2 is the variance conditional on the restricted model.

The general model was fitted and compared to various submodels by fixing certain parameters to specific values. For example, under a Mendelian model, transmission parameters are restricted to: $\tau(LL)=1$, $\tau(HL)=0.5$ and $\tau(HH)=0$, reflecting Mendelian segregation at a single locus with two alleles. A dominant model restricts $\mu HL=\mu LL$, while a recessive model restricts $\mu HL=\mu HH$. The distribution of types in the population was assumed to follow a Hardy-Weinberg equilibrium. A restricted model was compared to the general model using the likelihood ratio test. When models were not hierarchical, the Akaike's information criterion (AIC) was used to compare the fit of different models. For a given model, AIC=-2ln(L)+2*p (p=number of parameters estimated), and the model with the lowest AIC is considered to be the most parsimonious model (37).

Because of family selection through affected probands, correction for ascertainment bias was made by conditioning the phenotypes of family members on those of the affected probands and that of one extra affected individual per pedigree, so each pedigree contains two probands (i.e., 80 probands in 40 pedigrees) (25).

Segregation of apoB levels and LDL subfraction profiles in FCH

Previous segregation analyses on this data set demonstrated that both the distribution of dense LDL subfraction profiles (25) and the aggregation of elevated plasma apoB levels (26) appeared to have a genetic basis in these FCH families. The segregation of dense LDL subfraction profiles was most consistent with an autosomal recessive locus with a population frequency of 0.42 (data not shown) (25). In a separate segregation analysis of plasma apoB, the apoB concentrations were adjusted by linear regression for the influence of age, age^2, gender, BMI, smoking habits, and alcohol intake prior to the segregation analysis, comparable with procedures reported by others (35). Subsequently, a codominant model of inheritance plus family correlations $\rho(PO)=\rho(SS)=0.15\pm0.03$ and spouse correlation $\rho(SP)=0.14\pm0.07$, best explained the segregation of adjusted plasma apoB levels (data not shown) (26).

Correction of LDL subfraction profile for putative apoB genotype influence

To allow a more quantitative assessment of the impact of a putative Mendelian locus on a secondary trait, Hasstedt and Moll proposed to compute for each individual the probabilities for all possible genotypes under a Mendelian model for the primary trait, and

subsequently to use these probabilities to compute genotypic-specific means for the secondary trait (38).

In order to apply this method on the present data set, each individual was classified into their most probable genotype at the apoB locus (i.e., AA, AB, or BB) by segregation analysis of plasma apoB levels. The mean K value of those individuals with one of the apoB genotype probabilities $\geq 70\%$ were used to condition the K values for the predicted influence of apoB genotypes. By this arbitrary criterium (39), 53 individuals were assigned to represent apoB genotype AA, 144 to apoB genotype AB and 95 to apoB genotype BB (n=292 assigned, representing 47% of all subjects), with mean adjusted K values of 0.41, -0.46 and -1.69, respectively. Subsequently, the K values of all 623 subjects were conditioned by subtracting these apoB genotype specific mean K values of the individual K values. For subjects with prob(AA)=prob(AB) (0.41-0.46)/2 was subtracted from their K value, and for those with prob(AB)=prob(BB) (-0.46-1.69)/2 was subtracted. For those with missing plasma apoB values, (0.41-0.46-1.69)/3 was subtracted from the K values. Finally, the residual K values were used in the present segregation analysis.

RESULTS

Sample

566 Family members (i.e., 80 probands and 486 relatives) and 121 spouses from 40 FCH kindreds (consisting of 2 to 4 generations with pedigree size ranges from 7 to 104 family members, including spouses) participated. Because of lack of the required plasma volume (n=28) or technical errors (n=23), the LDL subfraction profiles of 51 subjects could not be determined. For an additional 13 subjects the apoB concentration was missing due to plasma sample unavailability. However, plasma lipid and lipoprotein levels of these subjects with missing apoB or LDL subfraction profile data were taken into account in order to support the diagnosis FCH. The present segregation analysis involved data of 623 subjects (299 men and 324 women). The mean lipid, (apo)lipoprotein levels and K values of the 80 probands, the FCH relatives and the spouses, adjusted for age, gender, BMI, and smoking habits are presented in Table 1. Due to the inclusion criteria, probands exhibited higher plasma lipid and lipoprotein levels than relatives and spouses. Also the plasma apoB concentration was higher and the K value was more negative in the probands compared with relatives and spouses. FCH relatives differed significantly from spouses on all parameters except for the LDL cholesterol concentration.

ApoB genotype assignment and effects on lipids, (apo)lipoproteins and K values

Of all 662 individuals (original 687 individuals minus 25 with missing data for apoB standardization) used in the segregation analysis of plasma apoB, one putative apoB genotype was more likely than the other two in 524 subjects (79%), whereas in 37 subjects (6%) two genotypes were equally likely. 101 Subjects (15%) could not be classified.

Table 1: Mean (±SD) adjusted[#] lipid and (apo)lipoprotein concentrations and K values in probands, FCH relatives and spouses of 40 FCH families.

	Probands	FCH relatives	Spouses
Number	80	480	102
Age (years)	54.6 ± 11.4	38.9 ± 16.8	50.0 ± 11.9*
BMI (kg/m^2)	26.7 ± 3.8	24.2 ± 3.7	25.5 ± 3.5*
Total plasma cholesterol[^]	6.43 ± 1.40	5.84 ± 1.07	5.58 ± 1.13*
Triglycerides[^]	2.58 ± 2.54	1.86 ± 1.47	1.18 ± 0.80*
VLDL cholesterol[^]	1.16 ± 1.32	0.77 ± 0.67	0.45 ± 0.41*
VLDL triglycerides[^]	1.85 ± 2.19	1.23 ± 1.21	0.67 ± 0.67*
HDL cholesterol[^]	1.12 ± 0.27	1.18 ± 0.29	1.31 ± 0.33*
LDL cholesterol[^]	4.16 ± 1.15	3.85 ± 0.98	3.91 ± 0.99
Apolipoprotein-B[B]	157.3 ± 42.9	133.4 ± 29.5	117.4 ± 27.1*
K value	-0.32 ± 0.29	-0.24 ± 0.26	-0.09 ± 0.23*

[#]values, except for age and BMI, were adjusted for the influence of age, gender, body mass index and smoking habits; [^]in mmol/l; [B]in mg/dl; BMI=body mass index; VLDL=very low density lipoprotein; HDL=high density lipoprotein; LDL=low density lipoprotein; *significant difference between FCH relatives and spouses.

The distribution of assigned apoB genotypes (i.e., AA, AB, or BB) for probands, relatives and spouses is presented in Table 2. In FCH probands and FCH relatives the 'high' apoB level genotype BB and the 'intermediate' apoB level genotype AB were more frequently assigned compared with spouses, whereas the 'low' apoB level genotype AA was more frequently assigned to spouses. Table 3 presents the lipids, (apo)lipoproteins and K value related to each apoB genotype. Age and BMI did not show significant differences between

genotypes. Compared with the apoB AA genotype, the apoB BB genotype corresponded with the highest plasma cholesterol, triglyceride, VLDL cholesterol, VLDL triglyceride and LDL cholesterol levels, and the lowest HDL cholesterol level.

Table 2: Distribution (n, (%)) of the assigned apolipoprotein-B level genotype (i.e , AA, AB, or BB), as provided by segregation analysis, for the probands, FCH relatives and spouses of 40 FCH families.

	Probands	FCH relatives	Spouses
Total number	80	480	102
ApoB AA genotype	9 (11 3%)	79 (16 5%)	42 (41 2%)
ApoB AA=AB genotype	2 (2,5%)	26 (5 4%)	2 (2 0%)
ApoB AB genotype	22 (27 5%)	225 (46 9%)	29 (28 4%)
ApoB AB=BB genotype	1 (1 3%)	6 (1 3%)	0 (0 0%)
ApoB BB genotype	31 (38 8%)	84 (17 5%)	3 (2 9%)
Unclassified	15 (18 8%)	60 (12 5%)	26 (25 5%)

ApoB=apolipoprotein-B, apoB AA genotype is associated with 'low' apoB levels, apoB AB genotype is associated with 'intermediate' apoB levels and apoB BB genotype is associated with 'high' apoB levels

Interestingly, the apoB BB genotype was also significantly associated with the lowest K value, representing more dense LDL subfraction profiles. The apoB AB genotype represented values for the parameters in between apoB AA and BB genotypes. Thus, the apoB BB genotype corresponded with a FCH lipid phenotype with elevated plasma lipids and lipoproteins, a decreased HDL cholesterol and a dense LDL subfraction profile. In the entire FCH population without spouses, the variability in crude plasma apoB concentration could account for 42% of the variability in LDL subfraction profiles, revealing a strong correlation (Spearman's correlation r=-0.65) between crude values for both variables (Figure 1). After adjustment for 'environmental' influences (see methods) of both apoB concentrations and K values, this correlation was still significant (r=-0.47) However, after adjustment of standardized K values for the predicted influence of apoB genotype, the correlation was completely disappeared (Spearman's correlation r=-0.08) This implicates that LDL subfraction profiles were adequately corrected for inferred genetical

influence of plasma apoB concentration.

Table 3: Mean (±SD) lipids, (apo)lipoproteins and K value by apoB level genotypes (i e , AA, AB, or BB) as designated by segregation analysis

	ApoB AA genotype	ApoB AB genotype	ApoB BB genotype
Number	130	276	118
Age (years)	44 6 ± 16 0	40 6 ± 17 4	45 2 ± 15 3#
BMI (kg/m^2)	24 9 ± 4 3	24 3 ± 3 7	24 8 ± 3 0
Total plasma cholesterol[A]	5 04 ± 1 00	5 67 ± 1 14	7 17 ± 1 21*
Triglycerides[A]	1 08 ± 0 51	1 51 ± 1 29	2 95 ± 2 05*
VLDL cholesterol[A]	0 35 ± 0 21	0 57 ± 0 54	1 38 ± 1 20*
VLDL triglycerides[A]	0 59 ± 0 40	0 96 ± 1 16	2 02 ± 1 71*
HDL cholesterol[A]	1 33 ± 0 35	1 21 ± 0 36	1 05 ± 0 28*
LDL cholesterol[A]	3 36 ± 0 95	3 90 ± 1 06	4 73 ± 1 16*
Apolipoprotein-B[B]	106 1 ± 24 5	127 1 ± 30 0	180 8 ± 34 7*
K value	-0 05 ± 0 21	-0 20 ± 0 29	-0 46 ± 0 24*

See 'Methods' for apoB level genotype designation procedure, [A]in mmol/l, [B]in mg/dl, VLDL=very low density lipoprotein, HDL=high density lipoprotein, LDL=low density lipoprotein, #significantly different from apoB AB genotype, *significantly different from apoB AA and AB genotype

Segregation of LDL subfraction profiles corrected for apoB genotype

Segregation analysis was carried out using the primary standardized variable K (25) adjusted for the genetic influence of apoB genotype. Table 4 presents parameter estimates for 13 different models of inheritance In a sporadic or unimodal model, the spouse, parent-offspring, and sibling correlations were examined to determine multifactorial or polygenic influences (models 1 to 4) The spouse correlation (ρ(SP)) was found to be insignificant (0.16 ± 0 12) and was therefore fixed at 0 in all subsequent models The parent-offspring correlation (ρ(PO)) and sibling correlation (ρ(SS)) were found to be equal (0 16±0 05) Therefore, all subsequent models assumed a single correlation among first-degree relatives (ρ(SS)=ρ(PO))

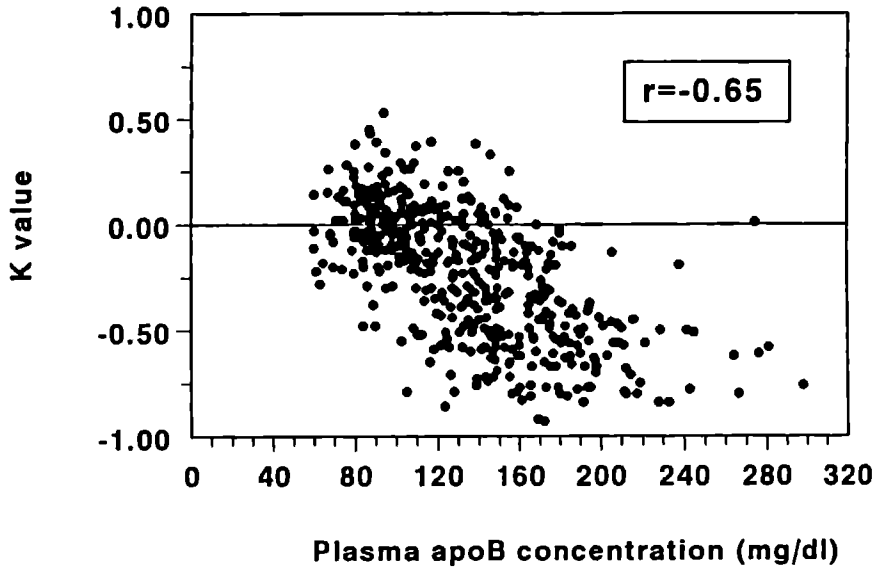

Figure 1: Correlation between crude plasma apoB concentrations and LDL density (i.e., the crude K value) observed in FCH relatives of 40 FCH families.

The sporadic models (models 1 to 4) and the non-genetic transmission models (models 11 and 12) fitted the data significantly worse than the unrestricted general model 13. This general model, with all variables unrestricted, provided evidence for major locus inheritance of parameter K, because transmission parameters $\tau(LL)=\{1.00\}$, $\tau(HL)=0.85\pm0.15$ and $\tau(HL)=0.09\pm0.07$, were roughly in accordance with values expected under Mendelian transmission: ($\tau(LL)=1$, $\tau(HL)=0.5$ and $\tau(HL)=0$), respectively, although $\tau(LL)$ had to be fixed at boundary. In order to examine the mode of inheritance, dominant models (models 5 and 6), recessive models (models 7 and 8), and codominant models (models 9 and 10) were fitted by fixing the transmission parameters at their Mendelian expectations. None of the Mendelian models could be rejected, because

Table 4: Segregation analysis of LDL subfraction profiles, adjusted# for the genotypic influence of Apo-B in 824 members of 40 kindreds

Model	q(L)	μ(LL)	μ(LH)	μ(HH)	σ^2	τ(LL)	τ(LH)	τ(HH)	ρ(SP)	ρ(PO)	ρ(SS)	-2ln(L)	AIC	Chi²	P-value
1. Sporadic	[1.0]	-0.00 ±0.05			1.46 ±0.09				[0.00]	[0.00]	[0.00]	1751.1	1755.1	51.4 (9 df)	<0.001
2. Sporadic + ρ(SP)	[1.0]	-0.00 ±0.05			1.47 ±0.09				0.16 ±0.12	[0.00]	[0.00]	1749.4	1755.4	45.7 (8 df)	<0.001
3. Sporadic + ρ(PO) + ρ(SS)	[1.0]	0.06 ±0.07			1.45 ±0.09				[0.00]	0.16 ±0.05	0.16 ±0.04	1722.2	1730.2	18.5 (7 df)	<0.01
4. Sporadic + ρ(SP) + ρ(PO/SS)	[1.0]	0.06 ±0.07			1.46 ±0.09				0.14 ±0.12	0.16 ±0.04	=ρ(PO)	1720.9	1728.9	17.2 (8 df)	<0.05
5. L dominant	0.03 ±0.01	-2.01 ±0.28	=μ(LL)	0.15 ±0.05	1.16 ±0.08	[1.0]	[0.5]	[0.0]	[0.00]	[0.00]	[0.00]	1713.3	1721.3	9.6 (7 df)	NS
6. L dominant + ρ(PO/SS)	0.03 ±0.01	-2.03 ±0.30	=μ(LL)	0.18 ±0.06	1.16 ±0.09	[1.0]	[0.5]	[0.0]	[0.00]	0.08 ±0.04	=ρ(PO)	1707.7	1717.7	4.0 (6 df)	NS
7. L recessive	0.26 ±0.05	-1.93 ±0.27	=μ(HH)	0.19 ±0.06	1.10 ±0.09	[1.0]	[0.5]	[0.0]	[0.00]	[0.00]	[0.00]	1716.5	1724.5	12.8 (7 df)	0.05<P<0.10
8. L recessive + ρ(PO/SS)	0.26 ±0.05	-1.92 ±0.28	=μ(HH)	0.22 ±0.07	1.10 ±0.10	[1.0]	[0.5]	[0.0]	[0.00]	0.10 ±0.04	=ρ(PO)	1709.8	1719.8	6.1 (6 df)	NS
9. Codominant	0.24 ±0.05	-2.01 ±0.27	-0.21 ±0.20	0.48 ±0.15	1.01 ±0.12	[1.0]	[0.5]	[0.0]	[0.00]	[0.00]	[0.00]	1712.7	1722.7	9.0 (6 df)	NS
10. Codominant + ρ(PO/SS)	0.28 ±0.05	-1.90 ±0.27	0.39 ±0.24	0.10 ±0.18	1.07 ±0.11	[1.0]	[0.5]	[0.0]	[0.00]	0.11 ±0.05	=ρ(PO)	1709.4	1721.4	5.7 (5 df)	NS
11. Environmental	0.41 ±0.07	-1.58 ±0.26	0.08 ±0.28	0.65 ±0.32	0.89 ±0.19	=q(L)	=q(L)	=q(L)	[0.00]	[0.00]	[0.00]	1741.5	1751.5	37.3 (6 df)	<0.001
12. Environmental + ρ(PO/SS)	0.38 ±0.26	-1.06 ±0.54	0.25 ±0.68	0.25 ±0.82	1.23 ±0.30	=q(L)	=q(L)	=q(L)	[0.00]	0.17 ±0.05	=ρ(PO)	1721.6	1733.6	17.9 (5 df)	<0.01
13. General	0.11 ±0.04	-1.92 ±0.33	0.41 ±0.18	0.09 ±0.12	1.08 ±0.14	(1.00)	0.85 ±0.12	0.09 ±0.07	0.15 ±0.12	0.11 ±0.06	0.10 ±0.06	1703.7	1725.7	Reference	

Values are presented as mean ± SE; # by subtracting the ApoB genotype-specific mean subfraction profiles of the K value;

[ ] = fixed externally; { } = fixed at boundary

their -2ln(L) values were very similar to that of the general model. Of the Mendelian models, those including the residual correlations provided the best fit of the data with the dominant model exhibiting the lowest AIC value. The residual correlation was used to estimate the polygenic component by the formula $[2\rho(PO)]^*[\sigma^2/\sigma_T{}^2]$ and was found to be 13% in the dominant model, 15% in the recessive model and 16% in the codominant model. Based on these results the Mendelian models (models 6, 8 and 10) provided the most parsimonious fit of the data, but discrimination between these models appeared to be impossible.

DISCUSSION

Based on data of these FCH families, both the aggregation of elevated apoB levels and the distribution of dense LDL subfraction profiles were shown to have a genetic basis in this lipid disorder. Because inheritance of the traits was investigated for each trait separately, the observed segregation of the individual traits may also have been caused by one genotype controlling both phenotypes. However, the segregation of dense LDL subfraction profiles corrected for putative apoB genotypes assigned by segregation analysis, still provided evidence for the presence of a Mendelian mechanism which exclusively controls the distribution of dense LDL subfractions profiles. Thus, it is likely that the presence of elevated plasma apoB levels and the predominance of small dense LDL are controlled by distinct genetic mechanisms in FCH.

Recently, we have demonstrated that a single major recessive locus controls the predominance of dense LDL subfraction profiles in these FCH families (25). This was consistent with the findings of Austin et al. (4). In contrast to an approach using a dichotomous classification of LDL subfraction pattern, or detection of the LDL peak particle diameter as performed by others (4,40,41), the great inter-individual variation in the LDL subfraction profiles would best be described by the continuous variable K. This K value is characterized by the relative contribution of all detected LDL subfractions to the total LDL subfraction profile (25,31). Compared with the general population, our analysis revealed a twice as high gene frequency in FCH families (25,31). Other reports support that also the physical and chemical properties of LDL particles in subjects with

FCH differ from those of normolipidemic controls (42). Many of these properties appeared to be independent of plasma triglyceride concentrations (43), supporting the specificity of dense LDL for this common lipid disorder.

The observed codominant Mendelian model of inheritance explaining the familial cluster of elevated plasma apoB levels in these FCH families (26) was reported previously in two other studies on FCH families (16,17). Also in families of patients with cardiovascular disease, which not completely met the FCH criteria, a codominant major locus effect was observed (14,35,44). Therefore, the genetic model for elevated plasma apoB level aggregation could explain both the genetic influence on plasma lipids and the increased incidence of cardiovascular disease in FCH families. So far, linkage analyses were not able to map a gene responsible for elevated apoB levels (45-47), although some markers showed some association (48). Interestingly, also linkage between markers of the apoB gene and the LDL subclass phenotype B could be excluded (49).

The presence of more than one locus controlling the plasma apoB levels and the LDL subfraction profiles has been previously suggested by observed bimodality of plasma apoB levels in FCH subjects with dense LDL (50). Furthermore, the reported lack of association between LDL subclass phenotype and apoB level genotype in a contingency analysis (17) is consistent with the present findings. Jarvik et al. demonstrated that a mixed Mendelian model of apoB inheritance was favored over a non-Mendelian 'major environmental' model after adjustment of apoB levels for LDL subclass phenotypes (17). Based on this, it was hypothesized that a multifactorial threshold model of inheritance of FCH, in which all subjects with the 'high' apoB genotype (i.e., 'BB') are over the threshold to develop FCH and in which subjects with the 'intermediate' or 'low' apoB genotype (i.e., 'AB' and 'AA', respectively) may be pushed over the threshold by having an additional allele for pattern B (i.e., dense LDL) (17). By contrast, the present study demonstrated inheritance of LDL subfraction profile after correction for the predicted genetic influence by apoB genotypes. This suggests inheritance of dense LDL subfraction profiles irrespective of the metabolic influence of elevated plasma apoB levels in FCH.

In assessing the validity of the results obtained from this study, several factors must be considered. First, the inability to rigorously distinguish those who are affected

from those who are not because of the absence of a specific marker so far. In addition, the fact that several defects may be involved in the phenotypic expression of FCH, predisposes for genetic heterogeneity in study samples (5,17,25). Large pedigrees, as used in this study, are expected to increase homogeneity. On the other hand, with a disorder frequency of 1 to 2% and probably additive genetic influences, the introduction of more than one causative trait may occur in a single family (17). Second, we adjusted a phenotype for the predicted genetic effects of another locus according to a method which was used before (17,38,39). This procedure necessitates a fairly valid assignment of the putative apoB genotype. Therefore, we only used those individuals with genotype probability above 70% to establish the influence on K values. The existence of different mean apoB levels among these apoB genotypes, which were also very similar to apoB genotype related concentrations predicted by the segregation analysis (26), supported the consistency of the genotype assignment. The significant influence of the different apoB genotypes on the K values was interpreted as metabolic relation between plasma apoB and LDL subfraction profile expression, although the direction of this influence could not be determined. In these FCH families, the metabolic relation between these two variables was also indicated by the original correlation between crude plasma apoB and crude K values, which, however, completely disappeared after the adjustment procedure of the K value for apoB genotype-specific influence. Therefore, the persistency of inherited susceptibility observed with the LDL subfractions adjusted for the influence of apoB genotypes is unlikely due to inadequate correction of LDL subfraction profiles.

Final proof of the major genes involved must come from the identification of susceptibility loci. Since the apoB locus itself could be excluded, other candidate genes involved in apoB overproduction have to be investigated. Possibly, defects preceding apoB secretion which may result in increased substrate delivery to hepatic apoB synthesizing cells (51), or defects in regulatory components of apoB synthesis such as the microsomal triglyceride transfer protein (52) can be considered. As far as plasma dense LDL is concerned, a locus near the LDL receptor on chromosome 19 has been linked to a predominance of these particles in plasma of normolipidemic subjects (53). This has been confirmed by a recent study which indicated a multilocus determination of dense LDL,

comprising the LDL receptor locus as well, but also the apoAI-CIII-AIV gene cluster, the cholesteryl ester transfer protein locus, and the manganese superoxide dismutase locus (41). The authors suggested that different genetically determined metabolic mechanisms may give rise to dense LDL particles, without finding evidence for genetic interaction with apoB production. Further studies have to establish these relations in hyperlipidemic families to better understand the atherogenic risk.

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Chapter 11

Summary and Conclusions

SUMMARY

Since the first description in 1973, familial combined hyperlipidemia (FCH) has kept lots of its secrets about etiology and the origin of associated premature vascular disease. Initially, FCH has been depicted as a monogenic autosomal inherited lipid disorder. However, mounting evidence suggests that FCH is more likely a heterogenous disorder comprehending key features of hyperapobetalipoproteinemia, the atherogenic lipoprotein phenotype, familial dyslipidemic hypertension and the insulin resistance syndrome. This heterogeneity in presenting lipid phenotype, still covered by the generally accepted diagnostic criteria for FCH, complicates the search for one or more common characteristics. In clinical practice, the absence of a specific marker for the disorder demands time-consuming family investigation before the diagnosis FCH is allowed in individuals. To have a chance to identify one or more specific marker(s) for the disorder, a combination of biochemic and genetic studies on apoB100 containing lipoproteins behaviours, including LDL subfractions, were performed in variable subsets from our data of 687 subjects from 40 FCH families. These families were gathered through well-known probands from the out-patient lipid clinic.

In **chapter 3a and 3b**, we evaluated the short term pharmacologic effects of two drugs with different mechanism of action (i.e., gemfibrozil and simvastatin) on plasma cholesterol and triglyceride concentrations, apoB100, apoCIII, apoAI and Lp(a) in 81 FCH patients. In a subset, also changes in LDL subfraction profile distribution and LDL oxidizability after treatment were studied. Considering plasma lipids, simvastatin reduced more effectively the plasma cholesterol concentration, whereas gemfibrozil reduced more effectively the plasma triglyceride concentration. However, the observed VLDL plus LDL cholesterol concentrations, both before and after both treatments, corresponded very well with those of total plasma apoB100 and thus the number of particles. The reduction in apoCIII level corresponded with the reduction in plasma triglyceride concentration in these FCH subjects. It was concluded that the total plasma apoB100 level represents the number of particles, which contain a constant cholesterol concentration in different FCH lipid phenotypes, despite treatment. The plasma apoCIII level may represent a variable amount of triglycerides per VLDL particle. Studies on LDL subfraction profiles before and after treatment showed that a decrease in triglyceride concentration corresponded with a reduction of the small dense LDL concentration, revealing a more buoyant LDL subfraction profile. Remarkedly, although triglyceride levels were frequently reduced to 'normal' values, the overall LDL subfraction profiles remained dense. This explains the observed small effect on LDL oxidizability, which is strongly related to LDL density.

Insulin resistance is associated with changes in lipid metabolism which are explained by overproduction of VLDL and impaired clearance of triglyceride rich lipoproteins due to increased hepatic availability of free fatty acids and reduced LpL activity, respectively. Because these features meet some of the criteria for FCH, insulin resistance may play a serious role in the etiology of FCH. So far, the presence of insulin resistance in FCH patients has been suggested only by indirect measurements. In **chapter 4** we compared the insulin sensitivity directly by means of the euglycemic hyperinsulinemic clamp technique of male non-obese, normotensive FCH patients with that of their non-affected relatives. In addition, an oral 75 gram glucose tolerance test (OGTT) was performed and forearm blood flow (FBF) was measured by venous occlusion plethysmography during the clamp. Although all participants had a normal glucose response after the glucose load, FCH patients showed higher baseline fasting C-peptide levels and hyperinsulinemia after OGTT. Mean whole body glucose uptake during the clamp was lower in FCH patients compared with the non-affected relatives. Interestingly, the glucose uptake correlated significantly with LDL density expressed by the so called K value, which we defined earlier. Furthermore, the FBF increased significantly in non-affected relatives but not in patients. Therefore, FCH patients which are characterized by a predominance of small dense LDL are insulin resistant as compared with their respective non-affected relatives exhibiting more buoyant LDL. This insulin resistance may partly be explained by a decreased insulin-induced vasodilation in skeletal muscle in FCH patients. For further research on the etiology of FCH, genes involved in the insulin resistance syndrome should be considered.

Hyperlipidemia due to reduced clearance of triglyceride rich lipoproteins is associated with both the FCH lipid phenotype and a metabolic mechanism wich provokes the formation of small dense LDL particles. In **chapter 5 and 6**, two factors: (i) decreased LpL activity and (ii) apoE polymorphism, involved in the clearance of triglyceride-rich lipoproteins were investigated in FCH families. (i) Compared with a population control group, the frequency of the recently described LpL Asn291→Ser mutation happened to be five to ten times higher in Dutch FCH patients (reported by others), and was found in 3 of 17 investigated FCH probands in our study. Using a pedigree-based maximum likelihood estimate on belonging family data, we demonstrated that, irrespective of age, gender and body mass index, the LpL(Asn291→Ser) mutation significantly affects the levels of plasma and VLDL triglycerides, and VLDL and HDL cholesterol, but not those of plasma and LDL cholesterol. These findings show that this LpL mutation is associated with elevated lipid levels, indicating that it may be one of the

genetic factors predisposing to FCH in the families studied. (ii) The impact of the apoE polymorphism on interindividual variation in plasma lipid, lipoprotein concentrations, and LDL subfraction profiles was studied in 201 well-defined FCH patients. In line with observations in other populations, carriers of the apo ε2 allele exhibited a substantial higher plasma triglyceride concentration and a lower LDL cholesterol level, while subjects with the apo ε4 allele had significant higher total plasma cholesterol and LDL cholesterol levels. Furthermore, it is likely that in FCH populations the apo ε4 allele is slightly over-represented. Although the apoE polymorphism by itself contributes significantly to the lipid and lipoprotein concentrations, and showes small effect on chemical composition of LDL from FCH subjects, it has no distinct effect on the distribution of LDL subfractions. This finding provides further evidence that a LDL subfraction profile consisting of small dense LDL particles is a characteristic feature in FCH patients, irrespective of apoE related metabolic influence.

The increased risk to develop cardiovascular disease in FCH, hard to explain by frequently only mild elevated plasma lipids, may be related to increased levels of small dense LDL particles, which are more prone to in vitro oxidative modification. In **chapter 7** we compared the in vitro resistance to copper induced oxidation of total LDL obtained from FCH relatives with either buoyant LDL or dense LDL. In addition, the LDL concentrations of antioxidant α-tocopherol and the coenzyme Q10 were measured and related to LDL oxidizability. It appeared that dense LDL particles from subjects with FCH are less resistant to in vitro oxidation if compared with buoyant LDL from normolipidemic relatives. Compared with subjects with an overall buoyant LDL subfraction profile, the redox status of coenzyme Q10 was reduced in subjects with an overall dense LDL subfraction profile, independently of the plasma cholesterol or triglyceride concentration. Therefore, a reduced redox status of coenzyme Q10 as observed in FCH patients, can be considered as an important indicator of already ongoing in vivo oxidative LDL modification. This observation increasingly connects small dense LDL to the presence of (premature) atherosclerose.

The plasma apoB is considered to be an estimate for the amount of particles determining one of the different FCH lipid phenotypes. Therefore, the familial plasma apoB level distribution was hypothesized to be a reliable reflection of the familial "FCH status". In **chapter 8** we describe the genetic coherence between different FCH lipid phenotypes represented by the plasma apoB level in 40 FCH families comprizing 623 individuals. By performing a segregation analysis on standardized plasma apoB levels, and after exclusion of one family with extreme apoB values, we demonstrated an autosomal

codominant mode of inheritance for plasma apoB levels. Although all Mendelian models of inheritance were prefered over non-Mendelian models, an autosomal dominant mode of inheritance, as initially described by Goldstein (1973), was rejected.

Since the occurence of small dense LDL in FCH patients is consistent irrespective of metabolic interferences, we performed analyses, described in **chapter 9**, to establish genetic influences on LDL subfraction profile distribution in 40 FCH families. Initially, LDL subfraction profiles of family members were described by a continuous variable K, which subsequently was standardized for variation due to age, sex, and hormonal status. By regression analysis it was shown that 60% of the variability of parameter K could be explained by lipid and lipoprotein levels. The remaining variability in parameter K was best explained by a major autosomal locus, recessively inherited, with a high population frequency, and an additional small polygenic component. Therefore, the clustering of atherogenic dense LDL subfraction profiles appear to have a genetic basis in these FCH families. However, since metabolic influences, i.e., elevated triglyceride levels, may jointly be responsible for the expression in LDL density, additional analyses were needed to unravel this relation between environmental, metabolic and genetic factors determining the LDL subfraction profile in FCH.

Because of differences in the observed genetic models for apoB and LDL subfraction profiles inheritance, it was deduced that the genetic mechanism underling apoB metabolism may differ from the mechanism involved in the expression of LDL particle density. In a final analysis, presented in **chapter 10**, This hypothesis was tested by evaluation of the inheritance of small dense LDL subfraction profiles (i.e., K values) after correction for the predicted genetic influence of a putative apoB locus, assigned by the previous segregation analysis. Segregation analysis of residual LDL subfraction profiles provided substantial evidence for a major locus inheritance pattern. Therefore, it was concluded that the previously observed inheritance of elevated plasma apoB levels indeed differed from the genetic influence on LDL subfraction distribution. These findings may support the search for molecular defects involved in the expression of the FCH phenotype associated with a preponderance of small dense LDL in patients.

GENERAL CONCLUSIONS

1. FCH is a metabolically and genetically heterogeneous lipid disorder.
2. Both factors that induce VLDL overproduction such as insulin resistance, or diminish the clearance of triglyceride-rich lipoproteins, such as decreased LpL activity or the apoE polymorphism, contribute to the lipid phenotypic presentation of

FCH.

3. Although not specific for this disorder, most FCH patients differ from their unaffected relatives by exhibiting a LDL subfraction profile predominated by small dense LDL particles, irrespective of their presenting lipid phenotype.
4. The increased cardiovascular risk in FCH may be explained by the increased suseptibility to oxidation of the small dense LDL particles as reflected by a in vivo reduced redox status of coenzyme Q10.
5. The plasma apoB concentration is an estimate for the number of VLDL and LDL particles involved in a FCH lipid phenotype. The inherited susceptibility of elevated plasma apoB levels in FCH demonstrates the genetic coherence of the disorder.
6. FCH is probably caused by a combination of genetic factors: those that influence the number of lipoprotein particles and those that determine the density of LDL particles.
7. So far, individual FCH patients can not be identified by the use of one specific marker.

Chapter 12

Samenvatting en Conclusies

SAMENVATTING

Hoewel de eerste beschrijving van familiaire gecombineerde hyperlipidemie (FCH) dateert uit 1973, bestaan er nog steeds veel onduidelijkheden rondom de etiologie en de geassocieerde vroegtijdige atherosclerose bij deze aandoening. Oorspronkelijk werd aangenomen dat FCH een monogenetische autosomaal overervende vetstofwisselingsstoornis was. Op grond van aanvullende gegevens nadien wordt tegenwoordig verondersteld dat FCH een heterogene aandoening is, waarbij karakteristieken van hyperapobetalipoproteïnemie, het "atherogene lipoproteïne fenotype", familiaire dyslipidemische hypertensie en het insulineresistentie syndroom kunnen worden waargenomen. Hoewel deze heterogene fenotypische presentatie nog steeds past binnen de algemeen aanvaarde diagnostische kriteria voor FCH, wordt het vinden van een specifieke marker voor de aandoening hierdoor bemoeilijkt. In de dagelijkse praktijk noodzaakt het gebrek aan zo'n specifieke marker tot tijdrovend familie-onderzoek voordat een correcte individuele diagnose kan worden gesteld. Met behulp van uitgebreid biochemisch en genetisch onderzoek naar het gedrag van apoB100 bevattende lipoproteïnen, inclusief LDL subfracties, werd getracht specifieke kenmerken van FCH te identificeren. Dit onderzoek werd verricht bij verschillende subgroepen van in totaal 687 patiënten en familieleden uit 40 FCH families, verzameld via probandi bekend van de lipidenpolikliniek.

In **hoofstuk 3a en 3b** werden de korte termijn farmacologische effecten van twee geneesmiddelen met een verschillend werkingsmechanisme (n.l. het fibraat gemfibrozil en de HMG-CoA reductaseremmer simvastatine) op de plasmaconcentraties van cholesterol, triglyceriden, apoB100, apoCIII, apoAI an Lp(a) geëvalueerd bij 81 FCH patiënten. In een subanalyse werden de veranderingen in LDL subfractieprofielen en de LDL oxideerbaarheid na behandeling bestudeerd. Simvastatine bleek de plasma cholesterolconcentratatie effectiever te verlagen dan gemfibrozil, terwijl de plasma triglyceride-concentratie sterker werd verlaagd door gemfibrozil. De VLDL plus LDL cholesterolconcentratie, zowel voor als na beide behandelingen, correspondeerde goed met de plasma apoB100 concentratie, een maat voor het aantal lipidendeeltjes. De verlaging van de apoCIII concentratie kwam goed overeen met de afname in plasma triglyceriden. Geconcludeerd werd dat het plasma apoB100 bij FCH het aantal deeltjes vertegenwoordigt. Deze bevatten een constante hoeveelheid cholesterol die niet wordt beïnvloed door de behandeling. Het plasma apoCIII kan gezien worden als een maat voor de hoeveelheid triglyceriden per VLDL deeltje. Bestudering van het LDL subfratieprofiel voor en na behandeling liet zien dat een afname van de triglyceridenconcentratie resulteerde in een afname van de hoeveelheid zware LDL subfracties. Opmerkelijk was de

bevinding dat bij normalisering van de plasma triglyceriden het LDL subfractiepatroon van deze FCH patienten bleef bestaan uit overwegend zware LDL subfracties. Dit verklaart waarschijnlijk het geringe effect van de beide behandelingen op de oxideerbaarheid van LDL, welke sterk gerelateerd is aan de LDL dichtheid.

Insulineresistentie wordt geassocieerd met veranderingen in het lipidenmetabolisme welke worden verklaard door overproductie van VLDL en een gestoorde klaring van triglyceriderijke lipoproteinen. Deze veranderingen worden toegeschreven aan een toename van de vrije vetzuurconcentratie in de levercel en een verminderde lipoproteine-lipase (LpL) activiteit. Omdat een deel van deze veranderingen ook wordt waargenomen bij FCH, zou insulineresistentie wel eens een belangrijke rol kunnen spelen in de etiologie van FCH. Tot nog toe werd de aanwezigheid van insulineresistentie bij FCH patienten alleen via indirecte bepalingen vastgesteld. In **hoofdstuk 4** werd middels de euglycemische hyperinsulinemische clamptechniek de gevoeligheid voor insuline bij mannelijke niet-obese normotensieve FCH patienten vergeleken met die van hun niet aangedane familieleden. Aanvullend werden de resultaten van een orale glucose tolerantietest (OGTT) geevalueerd en werd tijdens de clamp een meting van de onderarmsdoorbloeding (FBF) door middel van veneuze occlusiepletysmografie verricht. Hoewel alle deelnemers een normale glucoserespons tijdens de OGTT lieten zien, werd bij FCH patienten een hogere uitgangswaarde voor het C-peptide en een hyperinsulinemie na de OGTT waargenomen. De gemiddelde totale lichaamsopname van glucose tijdens de clamp was lager in FCH patienten vergeleken met hun niet aangedane familieleden. Interessant genoeg correleerde deze glucose opname tijdens de clamp met de individuele LDL dichtheid. Verder nam de FBF bij niet aangedane familieleden, in tegenstelling tot de FCH patienten, tijdens de clamp toe. Dus FCH patienten, gekarakteriseerd door een overmaat aan zware LDL deeltjes bleken vergeleken met hun niet aangedane familieleden met een lichter LDL subfractiepatroon insulineresistent te zijn. Deze insulineresistentie kon voor een deel verklaard worden door een afgenomen insuline gemedieerde vasodilatatie in de skeletspier bij FCH patienten. Bij verder onderzoek naar de etiologie van FCH zullen ook de genen die van belang zijn bij het insulineresistentiesyndroom moeten worden betrokken.

Hyperlipidemie veroorzaakt door een vertraagde klaring van triglyceriderijke lipoproteinen wordt geassocieerd met zowel het FCH fenotype als met metabole mechanismen die resulteren in de formatie van zware LDL subfracties. In **hoofdstuk 5 en 6** werden twee factoren die een rol spelen in de klaring van triglyceriderijke lipoproteinen (i) afgenomen LpL activiteit en (ii) invloeden van het apoE polymorfisme, onderzocht in FCH families. (i) Vergeleken met een controlegroep voor de algehele populatie komt een

recent beschreven LpL Asn291→Ser mutatie 5 tot 10 keer vaker voor in Nederlandse FCH patiënten (elders gerapporteerd) en werd deze in 3 van de 17 onderzochte FCH probandi uit onze studie teruggevonden. Middels een maximale 'likelihood' berekening op basis van de familiestructuur van deze 3 probandi bleek dat onafhankelijk van de leeftijd, het geslacht en de body mass index, deze LpL mutatie een significant invloed heeft op de concentratie van VLDL triglyceriden, VLDL cholesterol en HDL cholesterol. Deze bevinding laat zien dat de LpL Asn291→Ser mutatie leidt tot verhoogde lipidenwaarden, waardoor het een genetische factor kan zijn die predisponeerd voor FCH in de onderzochte families. (ii) De invloed van het apoE polymorfisme op initerindividuele variatie in lipiden, lipoproteïnen concentraties en LDL subfractiepatronen werd onderzocht bij 201 aangedane FCH patiënten. In overeenstemming met door anderen in verschillende populaties waargenomen effecten op lipidenconcentraties, hadden carriers van het apo $\epsilon 2$ allel een hogere plasma triglycerideconcentratie en een lagere LDL cholesterol-concentratie. Carriers van het apo $\epsilon 4$ allel hadden een significant hogere plasma en LDL cholesterolconcentratie. Verder werd een hogere frequentie van het apo $\epsilon 4$ allel in de FCH populatie aangetroffen. Ondanks de invloeden op plasma lipiden en -lipoproteïnen werd er slechts een marginale invloed op de LDL samenstelling en het LDL subfractiepatroon gevonden. Deze bevinding onderstreept dat onafhankelijk van door apoE genotypen gerelateerde metabole invloeden, een zwaar LDL subfractiepatroon FCH karakteriseert.

Het verhoogde risico op cardiovasculaire aandoeningen bij FCH, onvoldoende verklaard door de vaak slechts geringe lipidenverhoging, wordt mogelijk versterkt door de overmaat aan zware LDL subfracties, welke gevoeliger zijn voor oxidatieve modificatie in vitro. In **hoofdstuk 7** vergeleken we de in vitro resistentie tegen door koperionen geïnduceerde LDL cholesterol oxidatie bij FCH familieleden met een licht versus een zwaar LDL subfractiepatroon. Tevens werden de concentraties van de antioxidantia α-tocopherol en coenzyme Q10 in LDL deeltjes bepaald en gerelateerd aan de gemeten LDL oxidatiegevoeligheid. Het bleek dat, vergeleken met licht LDL van de normolipidemische familieleden, zware LDL deeltjes van FCH patiënten minder resistent waren tegen in vitro koperoxidatie. Vergeleken met familieleden met een licht LDL subfractiepatroon was de redoxstatus van het coenzym Q10 bij FCH patiënten met een zwaar LDL subfractiepatroon onafhankelijk van de lipidenconcentraties afgenomen. Deze afgenomen redoxstatus van coenzym Q10 kan gezien worden als een indicator voor reeds in vivo optredende LDL oxidatie. Deze gegevens onderbouwen de hypothese dat zwaar LDL eerder leidt tot oxidatieve modificatie van deze deeltjes, een proces dat geassocieerd wordt met (premature) atherosclerose.

De plasma apoB concentratie kan worden beschouwd als een schatting voor de hoeveelheid deeltjes verantwoordelijk voor één van de verschillende FCH lipidenfenotypen. Daarom werd verondersteld dat de verdeling van apoB concentraties over de verschillende familieleden een goede afspiegeling zou zijn van de "FCH status" in een familie. In **hoofstuk 8** beschrijven we de genetische samenhang tussen de verschillende FCH lipidenfenotypen, vertegenwoordigd door een plasma apoB concentratie, in 40 FCH families. Met behulp van een segregatie analyse van gestandaardiseerde apoB waarden, waarbij één familie met extreme apoB waarden was uitgesloten, werd een autosomaal codominant model voor overerving aangetoond. Hoewel alle Mendeliaanse modellen voor overerving prevaleerde boven de niet-Mendeliaanse modellen, werd een dominant model zoals in 1973 door Goldstein e.a. werd verondersteld, verworpen.

Omdat de aanwezigheid van een zwaar LDL subfractiepatroon bij FCH patiënten, onafhankelijk van metabole invloeden, een constant gegeven blijkt te zijn werd in **hoofdstuk 9** een analyse uitgevoerd om na te gaan of genetische invloeden het LDL subfractiepatroon bij FCH bepalen. Allereerst werden de LDL subfractiepatronen vertaald in een continue variabele K, welke vervolgens werd gestandaardiseerd voor variatie door leeftijd, geslacht en hormonale status. Door middel van regressie-analyse werd aangetoond dat 60 % van de variatie in K-waarde kon worden verklaard door variatie in lipiden- en lipoproteïnenwaarden. De resterende variatie kon na een segregatie analyse het beste worden verklaard door de aanwezigheid van een major autosomaal locus, dat recessief overerft en een hoge populatiefrequentie heeft in combinatie met een geringe additionele polygenetische component. Op grond van deze uitkomst wordt verondersteld dat de clustering van zware LDL subfractiepatronen in deze FCH families ook een genetische basis heeft. Echter omdat, blijkens de regressie-analyse, lipidenconcentraties het LDL subfractiepatroon mede bepalen waren aanvullende analyses noodzakelijk om de relatie tussen metabole factoren en genetische factoren, verantwoordelijk voor de uiteindelijke expressie, verder te ontrafelen.

Omdat in de onderzochte families het model voor de apoB overerving verschilde van het overervingsmodel voor de LDL subfractiepatronen, zou het genetische mechanisme verantwoordelijk voor het apoB metabolisme anders kunnen zijn dan het mechanisme betrokken bij de expressie in LDL dichtheid. In een finale analyse, gepresenteerd in **hoofdstuk 10**, werd deze hypothese getest door hernieuwde evaluatie van de LDL subfractiepatroon (K-waarde) overerving na correctie voor de voorspelde genetische invloed door het veronderstelde apoB locus. Segregatie analyse met de residuale K-waarde leverde overtuigend bewijs voor een resterend major locus effect. Op grond hiervan werd

geconcludeerd dat het eerder gevonden apoB overervingsmodel inderdaad verschilde van de genetische invloed op de LDL subfractiepatronen. Deze bevinding kan een bijdrage leveren bij de verdere zoektocht naar moleculaire defecten betrokken bij de expressie van het FCH lipidenfenotype dat wordt gekenmerkt door een overmaat aan zwaar LDL bij FCH patiënten.

ALGEMENE CONCLUSIES

1. FCH is een metabool en genetisch heterogene vetstofwisselingsstoornis.
2. Zowel factoren die VLDL overproductie induceren, zoals insulineresistentie, als factoren die leiden tot een vertraagde klaring van triglyceriderijke lipoproteïnen, zoals verminderde LpL activiteit en het apoE polymorfisme, dragen bij tot de presentatie van het FCH lipidenfenotype.
3. Hoewel niet specifiek voor deze aandoening, onderscheiden FCH patiënten zich van hun niet-aangedane familieleden door een zwaar LDL subfractiepatroon, onafhankelijk van het presenterende lipidenfenotype.
4. Het verhoogde risico op cardiovasculaire aandoeningen bij FCH kan worden verklaard door de toegenomen gevoeligheid voor oxidatie van zware LDL deeltjes, in vivo weerspiegeld door de afgenomen redoxstatus van het coenzym Q10.
5. De plasma apoB concentratie is een maat voor het aantal VLDL en LDL deeltjes dat betrokken is bij een FCH lipidenfenotype. De overerving van verhoogde apoB concentraties in FCH families geeft de genetische samenhang van de aandoening weer.
6. FCH wordt waarschijnlijk veroorzaakt door een combinatie van genetische factoren: namelijk die factoren die het aantal lipoproteïnendeeltjes bepalen en die factoren verantwoordelijk voor de dichtheid van LDL deeltjes.
7. Tot nog toe kunnen individuele FCH patiënten niet worden geïdentificeerd aan de hand van een specifieke marker.

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Hoewel niet de laatste pagina van dit boekje, is het hier geschrevene eindelijk het laatste loodje van dit proefschrift. Graag zou ik hierbij alle mensen willen bedanken die een bijdrage hebben geleverd aan de totstandkoming hiervan.

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CURRICULUM VITAE

De auteur van dit proefschrift werd geboren op 22 juli 1964 te Nijmegen. In 1982 behaalde hij het VWO-diploma aan de Nijmeegse Scholengemeenschap te Nijmegen. Na een colloquium doctum in de vakken natuurkunde en scheikunde aan de avondscholengemeenschap Cranenveldt te Nijmegen en het vervullen van zijn militaire dienstplicht werd in 1984 een aanvang gemaakt met de studie Geneeskunde aan de Katholieke Universiteit Nijmegen, alwaar op 31 augustus 1988 het doctoraalexamen en op 17 mei 1991 het artsexamen werd behaald. Voor de wetenschappelijke stage van de medische opleiding, onder leiding van Dr. P.M. Netten en Prof. Dr. W.H.L. Hoefnagels, getiteld "De invloed van de leeftijd op normale waarden voor cardiovasculaire reflextesten", ontving hij op 25 mei 1992 de Universitaire Prijs Bevordering Studiezin. Na werkzaam te zijn geweest als ambulance-arts te Andel en als arts-assistent niet in opleiding in het Streekziekenhuis te Bennekom, werd tussen februari 1993 en juli 1996 op de afdeling Algemene Interne Geneeskunde van het Academisch Ziekenhuis Nijmegen (hoofd: Prof. Dr. J.W.M. van der Meer), onder leiding van Prof. Dr. A.F.H. Stalenhoef, het wetenschappelijk onderzoek verricht dat heeft geleid tot dit proefschrift. Sinds 1 juli 1996 is hij in opleiding tot internist in de Kliniek voor Inwendige Ziekten van het Rijnstate Ziekenhuis te Arnhem (opleider: Dr. L. Verschoor). Hij is getrouwd met Karin Bos en heeft een dochtertje Marieke (1995).

Stellingen

behorende bij het proefschrift

Familial Combined Hyperlipidemia
metabolic and genetic aspects

in het openbaar te verdedigen
op donderdag 18 december 1997
des namiddags te 15.30 uur

door

Sebastian J. H. Bredie

I

In tegenstelling tot wat de pioniers in de beschrijving van familiaire gecombineerde hyperlipidemie vermoedden, kan op grond van de huidige inzichten worden gesteld dat deze vetstofwisselingsstoornis een *poly*genetische aandoening is

J L Goldstein et al , J Clin Invest 1973,52 1544-1568

Dit proefschrift

II

Bij de diagnostiek van familiaire gecombineerde hyperlipidemie wordt de waarde van het apolipoproteine-B gehalte overschat en de waarde van het LDL subfractiepatroon onderschat

Dit proefschrift

III

Bij het inschatten van het risico op prematuur vaatlijden is niet alleen de hoogte van het LDL cholesterolgehalte, maar ook de samenstelling van de LDL deeltjes van belang

J de Graaf et al Arterioscl Thromb 1991,11 298-306

Dit proefschrift

IV

Hoewel er voldoende aanwijzingen zijn dat het LDL subfractiepatroon genetisch wordt bepaald, dient het causale genlocus nog te worden bevestigd

Dit proefschrift

P M Nishina et al , Proc Natl Acad Sci U S A 1992,89 708-712

M J V Hoffer et al ongepubliceerde waarneming

V

Gedegen familie-onderzoek is aangewezen bij de opsporing van die personen die baat zullen hebben bij een preventieve cholesterolverlagende behandeling

Dit proefschrift

VI

Afwijkingen in cardiovasculaire reflextesten waarbij middels een finapress apparaat automatisch variaties in bloeddruk en hartslag worden gemeten, duiden op een gestoorde werking van het autonome zenuwstelsel

P M Netten, J M Boots, S J H Bredie et al , Clin Sci 1992,83 157-163

VII

Omdat de afgifte van endotheline-1 wordt gestimuleerd door geoxideerd LDL kunnen endothelinereceptorantagonisten in de toekomst een rol gaan spelen in de behandeling van atherosclerose door remming van chemoattractie van monocyten en proliferatie van gladde spiercellen in de vaatwand

H Haller et al Am J Physiol 1991,261 478-484

VIII

Bij een typisch klachtenpatroon voor coronaire insufficientie en kenmerkende afwijkingen op het electrocardiogram dient de diagnose cholelithiasis mede overwogen te worden

Eigen waarneming

IX

Zowel het ontbreken van adequate kinderopvang in grote overheidsinstellingen als een onvoldoende capaciteit in de zorg voor ouderen is anno 1997 onacceptabel

X

Hoewel het bij een verouderende samenleving maatschappelijk onverantwoord lijkt, dient voor de jongere generatie de mogelijkheid te worden geschapen om te kunnen werken in deeltijdverband, ter voorkoming van een dysbalans tussen werkenden en niet werkenden

XI

Hoge totaal cholesterolspiegels beschermen niet alleen LDL receptor 'knock-out' muizen, maar mogelijk ook hoog bejaarden ('85 plussers') tegen infecties met gram negatieve micro-organismen

M G Netea et al J Clin Invest 1996,97 1366-72

A Weverling-Rijnsburg et al Lancet 1997,350 1119-23

XII

Golfen is een sport voor mannen die willen knikkeren maar niet willen bukken

Youp van 't Hek